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(54) **TANDEM FLUORESCENT PROTEIN CONSTRUCTS**

TANDEM-FLUORESZENZPROTEINKONSTRUKTE

PRODUITS DE RECOMBINAISON PROTEIQUES FLUORESCENTS EN TANDEM

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Description

BACKGROUND OF THE INVENTION

[0001] Proteases play essential roles in many disease processes such as Alzheimer's, hypertension, inflammation, apoptosis, and AIDS. Compounds that block or enhance their activity have potential as therapeutic agents. Because the normal substrates of peptidases are linear peptides and because established procedures exist for making non-peptidic analogs, compounds that effect the activity of proteases are natural subjects of combinatorial chemistry. Screening compounds produced by combinatorial chemistry requires convenient enzymatic assays.

[0002] The most convenient existing assays for proteases are based on fluorescence resonance energy transfer from a donor fluorophore to a quencher placed at opposite ends of a short peptide chain containing the potential cleavage site. Knight CG, "Fluorimetric assays of proteolytic enzymes," *Methods in Enzymol.* (1995) 248:18-34. Proteolysis separates the fluorophore and quencher, resulting in increased intensity in the emission of the donor fluorophore. Existing protease assays use short peptide substrates incorporating unnatural chromophoric amino acids, assembled by solid phase peptide synthesis. However, solid phase synthesis poses certain problems of effort and expense.

Cubitt et al., TIBS 20, 448-455 describe that two GFP polypeptides (Y 66 H and S 65 C) are connected via a 25-residue spacer. WO 94/28166 refers to chemical fluorophores connected by an amino acid linker.

[0003] It is useful to perform enzymatic assays *in vivo*, in order to more closely mimic conditions in which intracellular proteases act. Conventional artificial substrates prepared by solid-phase synthesis would require microinjection into individual cells, which is impractical as a high-throughput screen. Also, short unfolded peptides are generally rapidly degraded by nonspecific mechanisms inside cells.

[0004] The Edans fluorophore is the current mainstay of existing fluorometric assays. Fluorophores with greater extinction coefficients and quantum yields are desirable. The Edans fluorophore often is coupled with a non-fluorescent quencher such as Dabcyl. However, assays performed with such agents rely on the absolute measurement of fluorescence from the donor. This amount of fluorescence intensily at a single wavelength is contaminated by other factors including turbidity or background absorbances of the sample, fluctuations in the excitation intensity, and variations in the absolute amount of substrate.

SUMMARY OF THE INVENTION

[0005] This invention provides tandem fluorescent protein constructs and methods for using them in enzymatic assays both *in vitro* and *in vivo*. Tandem fluorescent protein constructs comprise a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited by radiation,

characterized in that the linker moiety comprises a protease cleavage recognition site, and wherein cleavage of the linker by a protease results in a change in FRET between the donor and acceptor moieties,

and wherein the donor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acid substitutions,

a) Phe64Leu, Ser65Thr, Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala and Asn212Lys, or
b) Ser65Gly, Val68Leu, Ser72Ala and Thr203Tyr, or
c) Tyr66His and Tyr145Phe, or
d) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala and Asn212Lys,

and

the acceptor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acids substitutions,

a) Ser65Gly, Val68Leu, Ser72Ala and Thr203Tyr, or
b) Ser65Thr, Ser72Ala, Asn149Lys, Met153Thr and Ile167Thr;

or

wherein the donor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acid substitutions,

a) Tyr66His and Tyr145Phe, or

b) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala and Asn212Lys,

and
the acceptor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acids substitutions,

a) Ser65Cys, or
b) Ser65Thr.

The fluorescent protein moieties can be Aequorea-related fluorescent protein moieties, such as green fluorescent protein and blue fluorescent protein. In one aspect, the linker moiety comprises a cleavage recognition site for an enzyme, and is, preferably, a peptide of between 5 and 50 amino acids. In one embodiment, the construct is a fusion protein in which the donor moiety, the peptide moiety and the acceptor moiety are part of a single polypeptide.

[0006] This invention also provides recombinant nucleic acids coding for expression of tandem fluorescent protein constructs in which a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety are encoded in a single polypeptide. The invention also provides expression vectors comprising expression control sequences operatively linked to a recombinant nucleic acid coding for the expression of a tandem fluorescent protein construct, as well as host cells transfected with those expression vectors.

[0007] The tandem constructs of this invention are useful in assays for determining whether a sample contains an enzyme. The methods involve contacting the sample with a tandem fluorescent protein construct. The donor moiety is excited. Then the degree of fluorescence resonance energy transfer in the sample is determined. A degree of fluorescence resonance energy transfer that is lower than an expected amount indicates the presence of an enzyme. The degree of fluorescence resonance energy transfer in the sample can be determined as a function of the amount of fluorescence from the donor moiety, the amount of fluorescence from the acceptor donor moiety, the ratio of the amount of fluorescence from the donor moiety to the amount of fluorescence from the acceptor moiety or the excitation state lifetime of the donor moiety.

[0008] The assay also is useful for determining the amount of enzyme in a sample by determining the degree of fluorescence resonance energy transfer at a first and second time after contact between the enzyme and the tandem construct, and determining the difference in the degree of fluorescence resonance energy transfer. The difference in the degree of fluorescence resonance energy transfer reflects the amount of enzyme in the sample.

[0009] The invention also provides methods for determining the amount of activity of an enzyme in a cell. The methods involve providing a cell that expresses a tandem fluorescent protein construct, for example by transfecting the cell with an appropriate expression vector. The cell is exposed to light in order to excite the donor moiety. Then the degree of fluorescence resonance energy transfer in the cell is determined. The degree of fluorescence resonance energy transfer reflects to the amount of enzyme activity in the cell.

[0010] Similarly, the invention provides methods of determining the amount of activity of an enzyme in a sample from an organism. The methods involve providing a sample from an organism having a cell that expresses a tandem fluorescent protein construct. The donor moiety in the sample is excited. Then the degree of fluorescence resonance energy transfer in the sample is determined. The degree of fluorescence resonance energy transfer reflects the amount of enzyme activity in the cell.

[0011] The assay methods also can be used to determine whether a compound alters the activity of an enzyme, i. e., screening assays. The methods involve contacting a sample containing an amount of the enzyme with the compound and with a tandem fluorescent protein construct; exciting the donor moiety; determining the amount of enzyme activity in the sample as a function of the degree of fluorescence resonance energy transfer in the sample; and comparing the amount of activity in the sample with a standard activity for the same amount of the enzyme. A difference between the amount of enzyme activity in the sample and the standard activity indicates that the compound alters the activity of the enzyme.

[0012] Similar methods, are useful for determining whether a compound alters the activity of an enzyme in a cell. The methods involve providing first and second cells that express a tandem fluorescent protein construct; contacting the first cell with an amount of the compound; contacting the second cell with a different amount of the compound; exciting the donor moiety in the first and second cell; determining the degree of fluorescence resonance energy transfer in the first and second cells; and comparing the degree of fluorescence resonance energy transfer in the first and second cells. A difference in the degree of fluorescence resonance energy transfer indicates that the compound alters the activity of the enzyme.

[0013] Assays of the invention are also useful for determining and characterizing substrate cleavage sequences of proteases or for identifying proteases, such as orphan proteases. A library of fluorescent protein moieties each linked by a linker moiety can be generated using recombinant engineering techniques or synthetic chemistry techniques. Screening the members of the library can be accomplished by measuring a signal related to cleavage, such as fluo-

rescence energy transfer, after contacting the cleavage enzyme with each of the library members of the tandem fluorescent protein construct. A degree of fluorescence resonance energy transfer that is lower than an expected amount indicates the presence of a linker sequence that can be cleaved by the enzyme. The degree of fluorescence resonance energy transfer in the sample can be determined as a function of the amount of fluorescence from the donor moiety, the amount of fluorescence from the acceptor donor moiety, or the ratio of the amount of fluorescence from the donor moiety to the amount of fluorescence from the acceptor moiety or the excitation state lifetime of the donor moiety.

[0014] Libraries of fluorescent proteins can be expressed in cells and used to characterize the recognition motif of proteases expressed within cells, where the enzyme is in its native context. This method provides the additional advantage of assessing the specificity of any given linker sequence to cleavage by other enzymes other than the target enzyme. The methods consist of the generation of a library of recombinant host cells, each of which expresses a tandem fluorescent protein construct linked through a candidate linker substrate. Each cell is expanded into a clonal population that is genetically homogeneous and the degree of energy transfer is measured from each clonal population. Optionally, FRET can be measured before and at least one specified time after a known change in intracellular protease activity. A change in the degree of fluorescence resonance energy transfer demonstrates that the cell contains a tandem construct and linker sequence that can be cleaved by the enzyme activity in the cell. Such methods are particular suited to Fluorescent Activated Cell Sorter (FACS) clonal selection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

FIG. 1 depicts the nucleotide sequence and deduced amino acid sequence of a wild-type *Aequorea* green fluorescent protein.

FIG. 2 depicts a tandem construct of the invention involved in FRET.

FIG. 3 depicts fluorescence emission spectra of a composition containing a tandem S65C -- linker -- P4-3 fluorescent protein construct excited at 368 nm after exposure to trypsin for 0, 2, 5, 10 and 47 minutes.

FIG. 4 depicts fluorescence emission spectra intensity of a composition containing a tandem S65C -- linker -- P4-3 fluorescent protein construct excited at 368 nm after exposure to calpain for 0, 2, 6 and 15 minutes.

FIG. 5 depicts fluorescence emission spectra of a composition containing a tandem S65C -- linker -- P4 fluorescent protein construct excited at 368 nm after exposure to enterokinase for 0, 2, 20 and 144 minutes.

FIG. 6 depicts fluorescence emission spectra of a composition containing a tandem S65T -- linker -- W7 fluorescent protein construct excited at 432 nm before and after exposure to trypsin.

FIG. 7 depicts fluorescence emission spectra of a composition containing a tandem P4-3 -- linker -- W7 fluorescent protein construct excited at 368 nm before and after exposure to trypsin.

FIG. 8 depicts fluorescence emission spectra of a composition containing a tandem W1B -- linker -- 10c fluorescent protein construct excited at 433 nm before and after exposure to trypsin.

FIG. 9 depicts the time course of fluorescent ratio changes upon cleavage of a composition containing the tandem W1B -- linker -- 10c fluorescent protein construct measured at different protein concentrations after exposure to trypsin measured in a fluorescent 96 well plate reader.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0016] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, organic synthetic chemistry, and pharmaceutical formulation described below are those well known and commonly employed in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical formulation and delivery, and treatment of patients. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0017] "Moiety" refers to the radical of a molecule that is attached to another moiety. Thus, a "fluorescent protein moiety" is the radical of a fluorescent protein coupled to the linker moiety. By the same token, the term "linker moiety" refers to the radical of a molecular linker that is coupled to both the donor and acceptor protein moieties.

[0018] "Fluorescent protein" refers to any protein capable of fluorescence when excited with appropriate electromagnetic radiation. This includes fluorescent proteins whose amino acid sequences are either natural or engineered.

[0019] "Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. When the amino acids are a-amino acids, either the L-optical isomer or the D-optical isomer may be used. Additionally, unnatural amino acids, for example, b-alanine, phenylglycine and homoarginine are also meant to be included. Commonly encountered amino acids which are not gene-encoded may also be used in the present invention. All of the amino acids used in the present invention may be either the D- or L-isomer. The L-isomers are preferred. In addition, other peptidomimetics are also useful in the linker moieties of the present invention. For a general review see Spatola, A.F., in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[0020] "Naturally-occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

[0021] "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operatively linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

[0022] "Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding and non-coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0023] "Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

[0024] "Modulation " refers to the capacity to either enhance or inhibit a functional property of biological activity or process (e.g., enzyme activity or receptor binding); such enhancement or inhibition may be contingent on the occurrence of a specific event, such as activation of a signal transduction pathway, and/or may be manifest only in particular cell types.

[0025] The term "modulator" refers to a chemical compound (naturally occurring or non-naturally occurring), such as a biological macromolecule (e.g. nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Modulators are evaluated for potential activity as inhibitors or activators (directly or indirectly) of a biological process or processes (e.g., agonist, partial antagonist, partial agonist, antagonist, antineoplastic agents, cytotoxic agents, inhibitors of neoplastic transformation or cell proliferation, cell proliferation-promoting agents, and the like) by inclusion in screening assays described herein. The activities (or activity) of a modulator may be known, unknown or partial known. Such modulators can be screened using the methods described herein.

[0026] The term "test compound" refers to a compound to be tested by one or more screening method(s) of the invention as a putative modulator. Usually, various predetermined concentrations are used for screening such as .01 uM, .1 uM, 1.0 uM, and 10.0 uM. Test compound controls can include the measurement of a signal in the absence of the test compound or comparison to a compound known to modulate the target.

INTRODUCTION

[0027] It has been discovered that fluorescent proteins having the proper emission and excitation spectra that are brought into physically close proximity with one another can exhibit fluorescence resonance energy transfer ("FRET"). This invention takes advantage of that discovery to provide tandem fluorescent protein constructs in which two fluorescent protein moieties capable of exhibiting FRET are coupled through a linker to form a tandem construct. The protein moieties are chosen such that the excitation spectrum of one of the moieties (the acceptor moiety) overlaps with the emission spectrum of the excited protein moiety (the donor moiety). The donor moiety is excited by light of appropriate intensity within the donor's excitation spectrum. The donor then emits the absorbed energy as fluorescent light. The fluorescent energy it produces is quenched by the acceptor fluorescent protein moiety. FRET can be mani-

fested as a reduction in the intensity of the fluorescent signal from the donor, reduction in the lifetime of its excited state, and re-emission of fluorescent light at the longer wavelengths (lower energies) characteristic of the acceptor. When the linker that connects the donor and acceptor moieties is cleaved, the fluorescent proteins physically separate, and FRET is diminished or eliminated.

[0028] One can take advantage of the FRET exhibited by the tandem fluorescent protein constructs of the invention in performing enzymatic assays. An embodiment of this process is depicted in FIG. 2. A recombinant nucleic acid encodes a single polypeptide including a poly-histidinyl tag, a blue fluorescent protein donor moiety, a peptide linker moiety comprising a protease recognition site and a green fluorescent protein acceptor moiety. The nucleic acid can be expressed into a tandem fluorescent protein construct of the invention. In this example, a tandem construct contains a blue fluorescent protein (such as P4-3, **TABLE** I) as the donor moiety and a green fluorescent protein (such as S65C, **TABLE** I) as the acceptor moiety.

[0029] The construct is exposed to light at, for example, 368 nm, a wavelength that is near the excitation maximum of P4-3. This wavelength excites S65C only minimally. Upon excitation, some portion of the energy absorbed by the blue fluorescent protein moiety is transferred to the acceptor moiety through FRET. As a result of this quenching, the blue fluorescent light emitted by the blue fluorescent protein is less bright than would be expected if the blue fluorescent protein existed in isolation. The acceptor moiety (S65C) may re-emit the energy at longer wavelength, in this case, green fluorescent light.

[0030] After cleavage of the linker moiety by an enzyme, the blue and green fluorescent proteins physically separate and FRET is lost. Over time, as increasing amounts of the tandem construct are cleaved, the intensity of visible blue fluorescent light emitted by the blue fluorescent protein increases, while the intensity of visible green light emitted by the green fluorescent protein as a result of FRET, decreases.

[0031] The tandem fluorescent protein constructs of this invention are useful as substrates to study agents or conditions that cleave the linker. In particular, this invention contemplates tandem constructs in which the linker is a peptide moiety containing an amino acid sequence that is a cleavage site for a protease of interest. The amount of the protease in a sample is determined by contacting the sample with a tandem fluorescent protein construct and measuring changes in fluorescence of the donor moiety, the acceptor moiety or the relative fluorescence of both. In one embodiment, the tandem construct is a recombinant fusion protein produced by expression of a nucleic acid that encodes a single polypeptide containing the donor moiety, the peptide linker moiety and the acceptor moiety. Fusion proteins can be used for, among other things, monitoring the activity of a protease inside the cell that expresses the recombinant tandem construct. The distance between fluorescent proteins in the construct can be regulated based on the length of the linking moiety.

[0032] Advantages of tandem fluorescent protein constructs include the greater extinction coefficient and quantum yield of many of these proteins compared with those of the Edans fluorophore. Also, the acceptor in a tandem construct is, itself, a fluorophore rather than a non-fluorescent quencher like Dabcyl. Thus, the enzyme's substrate (i.e., the tandem construct) and products (i.e., the moieties after cleavage) are both fluorescent but with different fluorescent characteristics.

[0033] In particular, the substrate and cleavage products exhibit different ratios between the amount of light emitted by the donor and acceptor moieties. Therefore, the ratio between the two fluorescences measures the degree of conversion of substrate to products, independent of the absolute amount of either, the optical thickness of the sample, the brightness of the excitation lamp, the sensitivity of the detector, etc. Furthermore, the Aequorea-related fluorescent protein moieties tend to be protease resistant. Therefore, they are likely to survive as fluorescent moieties even after the linker moiety is cleaved.

II. TANDEM FLUORESCENT PROTEIN CONSTRUCTS

[0034] The tandem fluorescent protein constructs of the invention usually comprise three elements: a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties. The donor fluorescent protein moiety is capable of absorbing a photon and transferring energy to another fluorescent moiety. The acceptor fluorescent protein moiety is capable of absorbing energy and emitting a photon. The linker moiety connects the donor fluorescent protein moiety to the acceptor fluorescent protein moiety. In many instances the linker moiety will covalently connect the donor fluorescent protein moiety and the acceptor fluorescent protein moiety. It is desirable, as described in greater detail herein, to select a donor fluorescent protein moiety with an emission spectrum that overlaps with the excitation spectrum of an acceptor fluorescent protein moiety. In some embodiments of the invention the overlap in emission and excitation spectra will facilitate FRET, Such an overlap is not necessary, however, if intrinsic fluorescence is measured instead of FRET.

[0035] Green fluorescent proteins ("GFPs") of cnidarians, which act as their energy-transfer acceptors in bioluminescence, are used in the invention. A green fluorescent protein, as used herein, is a protein that fluoresces green light, and a blue fluorescent protein is a protein that fluoresces blue light. GFPs have been isolated from the Pacific

Northwest jellyfish, *Aequorea victoria*, the sea pansy, *Renilla reniformis*, and *Phialidium gregarium*. W.W. Ward et al., *Photochem. Photobiol.*, 35:803-808 (1982); L.D. Levine et al., *Comp. Biochem. Physiol.*, 72B:77-85 (1982).

[0036] A variety of *Aequorea*-related GFPs having useful excitation and emission spectra have been engineered by modifying the amino acid sequence of a naturally occurring GFP from *Aequorea victoria*. (D.C. Prasher et al., *Gene*, 111:229-233 (1992); R. Heim et al., *Proc. Natl. Acad. Sci., USA*, 91:12501-04 (1994); US-A-5 625 048, WO 96/23810, WO 98/06737). As used herein, a fluorescent protein is an *Aequorea*-related fluorescent protein if any contiguous sequence of 150 amino acids of the fluorescent protein has at least 85% sequence identity with an amino acid seauence, either contiguous or non-contiguous, from the wild type *Aequorea* green fluorescent protein of SEQ ID NO:2. More preferably, a fluorescent protein is an *Aequorea*-related fluorescent protein if any contiguous sequence of 200 amino acids of the fluorescent protein has at least 95% sequence identity with an amino acid sequence, either contiguous or non-contiguous, from the wild type Aequorea green fluorescent protein of SEQ ID NO:2. Similarly, the fluorescent protein may be related to *Renilla* or *Phialidium* wild-type fluorescent proteins using the same standards.

[0037] *Aequorea*-related fluorescent proteins include, for example, wild-type (native) *Aequorea victoria* GFP, whose nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences are presented in **FIG.** 1; and those *Aequorea*-related engineered versions described in **TABLE** I. Several of these, i.e., P4, P4-3, W7 and W2 fluoresce at a distinctly shorter wavelength than wild type.

TABLE I

Clone	Mutation(s)	Excitation max (nm)	Emission max (nm)	Extinct. Coefficient ($M^{-1}cm^{-1}$)	Quantum yield
Wild type	none	395 (475)	508	21,000 (7,150)	0.77
P4	Y66H	383	447	13,500	0.21
P4-3	Y66H; Y145F	381	445	14,000	0.38
W7	Y66W; N146I M153T V163A N212K	433 (453)	475 (501)	18,000 (17,100)	0.67
W2	Y66W; I123V Y145H H148R M153T V163A N212K	432 (453)	480	10,000 (9,600)	0.72
S65T	S65T	489	511	39,200	0.68
P4-1	S65T;M153A K238E	504 (396)	514	14,500 (8,600)	0.53
S65A	S65A	471	504		
S65C	S65C	479	507		
S65L	S65L	484	510		
Y66F	Y66F	360	442		
Y66W	Y66W	458	480		
10c	S65G; V68L S72A;T203Y	513	527		
W1B	F64L;S65T Y66W; N146I M153T V163A N212K	432(453)	476(503)		
Emerald	S65T; S72A N149K M153T I167T	487	508		
Sapphire	S72A; Y145F T203I	395	511		

[0038] The cloning and expression of yellow fluorescent protein from *Vibrio fischeri* strain Y-1 has been described by T.O. Baldwin et al., *Biochemistry* (1990) 29:5509-15. This protein requires flavins as fluorescent co-factors. The cloning of Peridinin-chlorophyll a binding protein from the dinoflagellate *Symbiodinium* sp. was described by B.J. Morris et al., *Plant Molecular Biology*, (1994) 24:673:77. One useful aspect of this protein is that it fluoresces in red. The cloning of phycobiliproteins from marine cyanobacteria such as *Synechococcus*, e.g., phycoerythrin and phycocyanin, is described in S.M. Wilbanks et al., *J. Biol. Chem.* (1993) 268:1226-35. These proteins require phycobilins as fluorescent co-factors, whose insertion into the proteins involves auxiliary enzymes. The proteins fluoresce at yellow to red wavelengths.

[0039] For FRET, the donor fluorescent protein moiety and the acceptor fluorescent protein moiety are selected so that the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited. One factor to be considered in choosing the fluorescent protein moiety pair is the efficiency of fluorescence resonance energy transfer between them. Preferably, the efficiency of FRET between the donor and acceptor moieties is at least 10%, more preferably at least 50% and even more preferably at least 80%. The efficiency of FRET can easily be empirically tested using the methods described herein and known in the art, particularly, using the conditions set forth in the **Examples.**

[0040] The efficiency of FRET is dependent on the separation distance and the orientation of the donor and acceptor moieties, as described by the Forster equation, the fluorescent quantum yield of the donor moiety and the energetic overlap with the acceptor moiety. Forster derived the relationship:

$$E = (F^0 - F)/F^0 = R_0^6/(R^6 + R_0^6)$$

where E is the efficiency of FRET, F and F^0 are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively, and R is the distance between the donor and the acceptor. R_0, the distance at which the energy transfer efficiency is 50%, is given (in Å) by

$$R_0 = 9.79 \times 10^3 (K^2 Q J n^{-4})^{1/6}$$

where K^2 is an orientation factor having an average value close to 0.67 for freely mobile donors and acceptors, Q is the quantum yield of the unquenched fluorescent donor, n is the refractive index of the intervening medium, and J is the overlap integral, which expresses in quantitative terms the degree of spectral overlap,

$$J = \int_0^\infty \varepsilon_\lambda F_\lambda \lambda^4 d\lambda / \int_0^\infty F_\lambda d\lambda$$

where ε_λ is the molar absorptivity of the acceptor in M^{-1} cm^{-1} and F_λ is the donor fluorescence at wavelength I measured in cm. Forster, T. (1948) *Ann.Physik* 2:55-75. Tables of spectral overlap integrals are readily available to those working in the field (for example, Berlman, I.B. *Energy transfer parameters of aromatic compounds*, Academic Press, New York and London (1973)).

[0041] The characteristic distance R_0 at which FRET is 50% efficient depends on the quantum yield of the donor i. e., the shorter-wavelength fluorophore, the extinction coefficient of the acceptor, i.e., the longer-wavelength fluorophore, and the overlap between the donor's emission spectrum and the acceptor's excitation spectrum. Calculated values of R_0 for P4-3 to S65T and S65C are both 4.03 nm because the slightly higher extinction coefficient of S65T compensates for its slightly longer emission wavelength. R. Heim et al., "Improved green fluorescence," *Nature* (1995) 373:663-664.

[0042] The efficiency of FRET between the two fluorescent proteins can also be adjusted by changing ability of the two fluorescent proteins to dimerize or closely associate. If the two fluorescent proteins are known or determined to closely associate, an increase or decrease in dimerization can be promoted by adjusting the length of the linker moiety between the two fluorescent proteins. Such dimerization can change Λ^2, R, J, and Q and dimerization changes directly affect the fluorescence spectra compared to undimerized protein. Consequently, for FRET aspects of the invention, the change in intrinsic fluorescence can be used to adjust the amount of FRET between the donor and the acceptor, as well as dimerization induced changes in FRET distances. Such dimerization induced changes in FRET distance can be optimized for maximal changes in FRET upon cleavage of a linker moiety by empirically determining the length of the linker moiety that produces the best FRET. Usually, such linkers will be comparable to a length of 14 to 30 amino acids.

[0043] The ability of two fluorescent proteins to dimerize could be increased by selecting amino acid positions that interact in the dimer and making changes of the amino acids at such positions that increase the hydrophobic or ionic interactions, or decrease the steric repulsions. Conversely, ability of two fluorescent proteins to dimerize could be decreased by selecting amino acid positions that interact in the dimer and making changes in the amino acids at such positions that decrease the hydrophobic or ionic interaction, or increase the steric repulsions. Thus, intramolecular interactions responsible for the association of fluorescent protein moieties in a tandem fluorescent protein or intermolecular interactions between two fluorescent proteins in free solution can be enhanced or attenuated.

[0044] For example, Aequorea derived fluorescent proteins and related proteins, especially at high concentrations of free protein, exist as dimers. The dimerization domain can be identified in the wild type protein using the crystal structure. Yang,F., et al The Molecular structure of Green Fluorescent Protein. Nature. Biotech. (1996) 14 1246-1251.

In the case of wildtype GFP, the hydrophobic amino acids, Ala 206, Leu 221, and Phe 223 interact during dimerization. The tendency of a tandem GFP (or two GFPs in free solution) to non-covalently associate at these positions could be increased by increasing the hydrophobicity of amino acids at positions 206 or 221, thereby increasing the strength of hydrophobic interactions between the two fluorescent proteins.

[0045] For example, replacement of Ala 206, or Leu 221 by any of the amino acids, Val, Ile or Phe would increase their hydrophobicity, and potentially strengthen the hydrophobic interaction between two GFPs. Alternatively, the amino acids could be changed to positively charged amino acids in one fluorescent protein (for example lys or Arg) and to negatively charged amino acids in the second fluorescent protein of the construct (for example Glu or Asp) thereby creating additional electrostatic interactions between two GFPs. Similarly the amino acids Tyr 39, Glu 142, Asn 144, Asn 146, Ser 147, Asn 149, Tyr 151, Arg 168, Asn 170, Glu 172, Tyr 200, Ser 202, Gln 204 and Ser 208 could be changed according to the methods described herein to enhance intramolecular interactions between tandem fluorescent proteins or intermolecular interactions between to GFPs in free solution.

[0046] The length of the linker moiety is chosen to optimize both FRET and the kinetics and specificity of enzymatic cleavage. The average distance between the donor and acceptor moieties should be between about 1 nm and about 10 nm, preferably between about 1 nm and about 6 nm, and more preferably between about 1 nm and about 4 nm. If the linker is too short, the protein moieties may sterically interfere with each other's folding or with the ability of the cleavage enzyme to attack the linker. In embodiments of the invention where dimerization is desired the linker length will typically be a length comparable the length of at least 12 amino acids, preferably at least 18 amino acids and more preferably at least 24 amino acids. Only in rare instances will the linker length be longer than the length of about 40 to 50 amino acids.

[0047] The effect of linker length on the ability of tandemly linked fluorescent proteins to become fluorescent was determined for a modified GFP tandem protein, as shown in **TABLE II.** The modified GFP tandem protein was expressed in bacteria and grown at 37(C.

TABLE II

Linker Length in amino acids	Fluorescence of 1st Fluorescent protein (Sapphire)	Fluorescence of 2nd Fluorescent protein (10C)
12	6.8×10^4	6.2×10^4
14	8.9×10^4	8.4×10^4
16	1.1×10^5	1.0×10^5
18	1.3×10^5	1.2×10^5
20	1.5×10^5	1.4×10^5
22	2.9×10^5	1.6×10^5
24	1.1×10^6	7.8×10^4
25	2.0×10^6	1.2×10^6

[0048] Tandem fluorescent proteins of the invention comprising the general form Sapphire - linker - 10C (10C is also known as Topaz) were expressed in the bacterial cells JM109 (DE3). The linker moiety was constructed with variable numbers of amino acids to evaluate the influence of linker size on fluorescence development. The linker sequences of **TABLE II** are described as SEQ ID NO.: 26 to 31, respectively. The composition of the 25 amino acid linker is identical to that used in the tandem fluorescent protein constructs in the Examples. After overnight growth at 37(C the bacterial colonies were examined to determine their relative fluorescence by resuspension in PBS after normalization for the number of bacteria present by measuring the optical density at 600 nm.

[0049] When the intramolecular dimerization of a tandem fluorescent protein construct is preferred, the three dimensional structure and flexability of the linker should permit the fluorescent protein moieties to associate. When the linker moiety contains a cleavage site, the length of the linker can be between about 5 and about 50 amino acids and more preferably between about 12 and 30 amino acids. Longer linkers may create too many sites which are vulnerable to attack by enzymes other than the one being assayed.

[0050] To optimize the efficiency and detectability of FRET within the tandem fluorescent protein construct, several factors need to be balanced. The emission spectrum of the donor moiety should overlap as much as possible with the excitation spectrum of the acceptor moiety to maximize the overlap integral J. Also, the quantum yield of the donor moiety and the extinction coefficient of the acceptor should likewise be as high as possible to maximize R_0. However, the excitation spectra of the donor and acceptor moieties should overlap as little as possible so that a wavelength region can be found at which the donor can be excited efficiently without directly exciting the acceptor. Fluorescence arising from direct excitation of the acceptor is difficult to distinguish from fluorescence arising from FRET. Similarly, the emission spectra of the donor and acceptor moieties should overlap as little as possible so that the two emissions

can be clearly distinguished. High fluorescence quantum yield of the acceptor moiety is desirable if the emission from the acceptor is to be measured either as the sole readout or as part of an emission ratio. In a preferred embodiment, the donor moiety is excited by ultraviolet (<400 nm) and emits blue light (<500 nm), whereas the acceptor is efficiently excited by blue but not by ultraviolet light and emits green light (>500 nm), for example, P4-3 and S65C.

[0051] In the tandem constructs of the invention, the donor and acceptor moieties are connected through a linker moiety. The linker moiety includes a cleavage recognition site specific for a protease. A cleavage site in the linker moiety is useful because when a tandem construct is mixed with the cleavage agent, the linker is a substrate for cleavage by the cleavage agent. Rupture of the linker moiety results in separation of the fluorescent protein moieties that is measurable as a change in FRET.

[0052] The cleavage agent of interest is a protease and the linker comprises a peptide containing a cleavage recognition sequence for the protease. A cleavage recognition sequence for a protease is a specific amino acid sequence recognized by the protease during proteolytic cleavage. In particular, the linker can contain any of the amino acid sequences in **TABLE III.** The sites are recognized by the enzymes as indicated and the site of cleavage is marked by a hyphen. Other protease cleavage sites also are known in the art and can be included in the linker moiety.

TABLE III

Protease	Sequence
HIV-1 protease	SQNY-PIVQ (SEQ ID NO:3)
	KARVL-AEAMS (SEQ ID NO:4)
Prohormone convertase	PSPREGKR-SY (SEQ ID NO:5)
Interleukin-1b-converting enzyme	YVAD-G (SEQ ID NO:6)
Adenovirus endopeptidase	MFGG-AKKR (SEQ ID NO:7)
Cytomegalovirus assemblin	GVVNA-SSRLA (SEQ ID NO:8)
Leishmanolysin	LIAY-LKKAT (SEQ ID NO:9)
b-Secretase for amyloid precursor protein	VKM-DAEF (SEQ ID NO:10)
Thrombin	FLAEGGGVR-GPRVVERH (SEQ ID NO:11)
Renin and angiotensin-converting enzyme	DRVYIHPF-HL-VIH (SEQ ID NO:12)
	KPALF-FRL (SEQ ID NO:13)
Cathepsin D	QPLGQTSLMK-RPPGFSPFR-SVQVMKT QEGS (SEQ ID NO:14)
Kininogenases including kallikrein	

See, e.g., Matayoshi et al. (1990) *Science* 247:954, Dunn et al. (1994) *Meth. Enzymol.* 241:254, Seidah & Chretien (1994) *Meth. Enzymol.* 244:175, Thornberry (1994) Meth. Enzymol. 244:615, Weber & Tihanyi (1994) *Meth. Enzymol.* 244:595, Smith et al. (1994) *Meth. Enzymol.* 244:412, Bouvier et al. (1995) *Meth. Enzymol.* 248:614, Hardy et al. (1994) in Amyloid Protein Precursor in Development, Aging, and Alzheimer's Disease, ed. C.L. Masters et al. pp. 190-198.

[0053] In the case of a known protease with cleavage activity of unknown or partially defined specificity, a library of linker sequences can be used in place of a predetermined linker sequence in the tandem fluorescent protein construct

in order to determine the sequences cleaved by a protease. The method can be used with a recombinant protease constructed with a novel cleavage specificity. This method can also be used to determine the specificity of cleavage of an orphan protein that reveals sequence homology to a known protease structure or group of proteases.

[0054] In one embodiment, a genetically engineered library of tandem fluorescent protein constructs having different linkers can be used to define the function of an orphan protease. Optionally, the orphan protease, especially to if is thought to be expressed relatively inactive precursor, can be coexpressed with the tandem fluorescent protein construct. The protease may also be coexpressed with the tandem construct and under the control of an inducable promoter.

[0055] As used herein, a "library" refers to a collection containing at least 5 different members, preferably at least 100 different members and more preferably at least 200 different members. Each member of a tandem fluorescent substrate library comprises 2 tandemly linked fluorescent protein moieties separated by a peptide linker moiety of variable amino acid composition. The library can be chemically synthesized, which is particularly desirable if d-amino acids are to be included. In most instances, however, the library will be expressed in bacteria or a mammalian cell.

[0056] For example, the library can contain linkers with a diverse collection of amino acids. Alternatively, the library can contain variable peptide moieties in which only a few, e.g., one to ten, amino acid positions are varied, but in which the probability of substitution is very high.

[0057] Preferably, libraries of tandem fluorescent protein candidate substrates are created by expressing protein from libraries of recombinant nucleic acid molecules having expression control sequences operatively linked to nucleic acid sequences that code for the expression of different fluorescent protein candidate substrates. Methods of making nucleic acid molecules encoding a diverse collection of peptides are described in, for example, U.S. patent 5,432,018 (Dower et al.), U.S. patent 5,223,409 (Ladner et al.) and International patent publication WO 92/06176 (Huse et al.).

[0058] For expression of tandem fluorescent protein candidate substrates, recombinant nucleic acid molecules are used to transfect cells, such that a cell contains a member of the library. This produces, in turn, a library of host cells capable of expressing a library of different fluorescent protein candidate substrates. The library of host cells is useful in the screening methods of this invention.

[0059] In one method of creating such a library, a diverse collection of oligonucleotides are combined to create polynucleotides encoding peptides having a desired number of amino acids for the linker moiety. The oligonucleotides preferably are prepared by chemical synthesis. The polynucleotides encoding peptide linker moiety of variable composition can then be ligated to the 5' or 3' end of a nucleic acid encoding one of the tandem fluorescent protein moieties, using methods known in the art. This creates a recombinant nucleic acid molecule coding for the expression of a fluorescent protein candidate substrate having a variable linker peptide moiety fused to the amino or carboxy- terminus of one of the tandem fluorescent proteins. This recombinant nucleic acid molecule is then inserted into an expression vector in which the second fluorescent has already been inserted to create a recombinant nucleic acid molecule comprising expression control sequences operatively linked to the sequences encoding the tandemly repeated fluorescent proteins separated by the linker moieties.

[0060] To generate the collection of oligonucleotides which forms a series of codons encoding a random collection of amino acids that is ultimately cloned into the vector, a codon motif is used, such as $(NNK)_x$, where N may be A, C, G, or T (nominally equimolar), K is G or T (nominally equimolar), and x is the desired number of amino acids in the peptide moiety, e.g., 15 to produce a library of 15-mer peptides. The third position may also be G or C, designated "S". Thus, NNK or NNS (i) code for all the amino acids, (ii) code for only one stop codon, and (iii) reduce the range of codon bias from 6:1 to 3:1. The expression of peptides from randomly generated mixtures of oligonucleotides in appropriate recombinant vectors is discussed in Oliphant et al., *Gene* 44:177-183 (1986).

[0061] An exemplified codon motif $(NNK)_6$ produces 32 codons, one for each of 12 amino acids, two for each of five amino acids, three for each of three amino acids and one (amber) stop codon. Although this motif produces a codon distribution as equitable as available with standard methods of oligonucleotide synthesis, it results in a bias for amino acids encoded by two or three alternative codons.

[0062] An alternative approach to minimize the bias against one-codon residues involves the synthesis of 20 activated tri-nucleotides, each representing the codon for one of the 20 genetically encoded amino acids. These are synthesized by conventional means, removed from the support but maintaining the base and 5-HO-protecting groups, and activating by the addition of 3'O-phosphoramidite (and phosphate protection with beta-cyanoethyl groups) by the method used for the activation of mononucleosides, as generally described in McBride and Caruthers, *Tetrahedron Letters* 22:245 (1983). Degenerate "oligocodons" are prepared using these trimers as building blocks. The trimers are mixed at the desired molar ratios and installed in the synthesizer. The ratios will usually be approximately equimolar, but may be a controlled unequal ratio to obtain the over- to under-representation of certain amino acids coded for by the degenerate oligonucleotide collection. The condensation of the trimers to form the oligocodons is done essentially as described for conventional synthesis employing activated mononucleosides as building blocks. See generally, Atkinson and Smith, *Oligonucleotide Synthesis*, M.J. Gait, ed. p35-82 (1984). Thus, this procedure generates a population of oligonucleotides for cloning that is capable of encoding an equal distribution (or a controlled unequal distribution) of the possible peptide sequences.

[0063] Because protease cleavage recognition sequences generally are only a few amino acids in length, the linker moiety can include the recognition sequence within flexible spacer amino acid sequences, such as GGGGS (SEQ ID NO:15). For example, a linker moiety including a cleavage recognition sequence for Adenovirus endopeptidase could have the sequence GGGGGGSMFG GAKKRSGGGG GG (SEQ ID NO:16).

[0064] This invention contemplates tandem fluorescent protein constructs produced in the form of a fusion protein by recombinant DNA technology as well as constructs produced by chemically coupling fluorescent proteins to a linker. In either case, the fluorescent proteins for use as donor or acceptor moieties in a tandem construct of the invention preferably are produced recombinantly.

[0065] Recombinant production of fluorescent proteins involves expressing nucleic acids having sequences that encode the proteins. Nucleic acids encoding fluorescent proteins can be obtained by methods known in the art. For example, a nucleic acid encoding the protein can be isolated by polymerase chain reaction of cDNA from *A. vctoria* using primers based on the DNA sequence of *A. victoria* green fluorescent protein, as presented in **FIG. 1**. PCR methods are described in, for example, U.S. Pat. No. 4,683,195; Mullis et al. (1987) *Cold Spring Harbor Symp. Quant. Biol.* 51: 263; and Erlich, ed., *PCR Technology,* (Stockton Press, NY, 1989). Mutant versions of fluorescent proteins can be made by site-specific mutagenesis of other nucleic acids encoding fluorescent proteins, or by random mutagenesis caused by increasing the error rate of PCR of the original polynucleotide with 0.1 mM $MnCl_2$ and unbalanced nucleotide concentrations. See, e.g., WO 96/23810.

[0066] The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. Sambrook et al., *Molecular Cloning -- A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1989) and *Current Protocols in Molecular Biology,* F.M. Ausubel et al., eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (most recent Supplement).

[0067] Nucleic acids used to transfect cells with sequences coding for expression of the polypeptide of interest generally will be in the form of an expression vector including expression control sequences operatively linked to a nucleotide sequence coding for expression of the polypeptide. As used, the term "nucleotide sequence coding for expression of" a polypeptide refers to a sequence that, upon transcription and translation of mRNA, produces the polypeptide. This can include sequences containing, e.g., introns. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are "operatively linked" to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons.

[0068] Recombinant fluorescent protein can be produced by expression of nucleic acid encoding the protein in *E. coli.* The fluorophore of Aequorea-related fluorescent proteins results from cyclization and oxidation of residues 65-67. *Aequorea*-related fluorescent proteins are best expressed by cells cultured between about 20° C and 30° C. After synthesis, these enzymes are stable at higher temperatures (e.g., 37° C) and can be used in assays at those temperatures.

[0069] The construct can also contain a tag to simplify isolation of the tandem construct. For example, a polyhistidine tag of, e.g., six histidine residues, can be incorporated at the amino terminal end of the fluorescent protein. The polyhistidine tag allows convenient isolation of the protein in a single step by nickel-chelate chromatography.

A. Recombinant Nucleic Acids Encoding Tandem Construct Fusion Proteins

[0070] In a preferred embodiment, the tandem construct is a fusion protein produced by recombinant DNA technology in which a single polypeptide includes a donor moiety, a peptide linker moiety and an acceptor moiety. The donor moiety can be positioned at the amino-terminus relative to the acceptor moiety in the polypeptide. Such a fusion protein has the generalized structure: (amino terminus) donor fluorescent protein moiety -- peptide linker moiety -- acceptor fluorescent protein moiety (carboxy terminus). Alternatively, the donor moiety can be positioned at the carboxy-terminus relative to the acceptor moiety within the fusion protein. Such a fusion protein has the generalized structure: (amino terminus) acceptor fluorescent protein moiety -- peptide linker moiety -- donor fluorescent protein moiety (carboxy terminus). The invention also envisions fusion proteins that contain extra amino acid sequences at the amino and/or carboxy termini, for example, polyhistidine tags.

[0071] Thus, tandem constructs encoded by a recombinant nucleic acid include sequences coding for expression of a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety. The elements are selected so that upon expression into a fusion protein, the donor and acceptor moieties exhibit FRET when the donor moiety is excited.

[0072] The recombinant nucleic acid can be incorporated into an expression vector comprising expression control

sequences operatively linked to the recombinant nucleic acid. The expression vector can be adapted for function in prokaryotes or eukaryotes by inclusion of appropriate promoters, replication sequences, markers, etc.

[0073] The expression vector can be transfected into a host cell for expression of the recombinant nucleic acid. Host cells can be selected for high levels of expression in order to purify the tandem construct fusion protein. *E. coli* is useful for this purpose. Alternatively, the host cell can be a prokaryotic or eukaryotic cell selected to study the activity of an enzyme produced by the cell. In this case, the linker peptide is selected to include an amino acid sequence recognized by the protease. The cell can be, e.g., a cultured cell or a cell *in vivo*.

[0074] A primary advantage of tandem construct fusion proteins is that they are prepared by normal protein biosynthesis, thus completely avoiding organic synthesis and the requirement for customized unnatural amino acid analogs. The constructs can be expressed in *E. coli* in large scale for *in vitro* assays. Purification from bacteria is simplified when the sequences include polyhistidine tags for one-step purification by nickel-chelate chromatography. Alternatively, the substrates can be expressed directly in a desired host cell for assays *in situ*, which is particularly advantageous if the proteases of interest are membrane-bound or regulated in a complex fashion or not yet abundant as purified stable enzymes. No other generalizable method for continuous nondestructive assay of protease activity in living cells or organisms presently exists.

III. ENZYMATIC ASSAYS USING TANDEM FLUORESCENT PROTEIN CONSTRUCTS

[0075] Tandem fluorescent protein constructs are useful in enzymatic assays. These assays take advantage of the fact that cleavage of the linker moiety and separation of the fluorescent moieties results in a measurable change in FRET. Methods for determining whether a sample has activity of an enzyme involve contacting the sample with a tandem fluorescent protein construct in which the linker moiety that couples the donor and acceptor moieties contains a cleavage recognition site specific for the enzyme. Then the donor moiety is excited with light in its excitation spectrum. If the linker moiety is cleaved, the donor and acceptor are free to drift apart, increasing the distance between the donor and acceptor and preventing FRET. Then; the degree of FRET in the sample is determined. A degree of FRET that is lower than the amount expected in a sample in which the tandem construct is not cleaved indicates that the enzyme is present.

[0076] The amount of activity of an enzyme in a sample can be determined by determining the degree of FRET in the sample at a first and second time after contact between the sample and the tandem construct, determining the difference in the degree of FRET. The amount of enzyme in the sample can be calculated as a function of the difference in the degree of FRET using appropriate standards. The faster or larger the loss of FRET, the more enzyme activity must have been present in the sample.

[0077] The degree of FRET can be determined by any spectral or fluorescence lifetime characteristic of the excited construct, for example, by determining the intensity of the fluorescent signal from the donor, the intensity of fluorescent signal from the acceptor, the ratio of the fluorescence amplitudes near the acceptor's emission maxima to the fluorescence amplitudes near the donor's emission maximum, or the excited state lifetime of the donor.

[0078] For example, cleavage of the linker increases the intensity of fluorescence from the donor, decreases the intensity of fluorescence from the acceptor, decreases the ratio of fluorescence amplitudes from the acceptor to that from the donor, and increases the excited state lifetime of the donor.

[0079] Preferably, changes in the degree of FRET are determined as a function of the change in the ratio of the amount of fluorescence from the donor and acceptor moieties, a process referred to as "ratioing." Changes in the absolute amount of substrate, excitation intensity, and turbidity or other background absorbances in the sample at the excitation wavelength affect the intensities of fluorescence from both the donor and acceptor approximately in parallel. Therefore the ratio of the two emission intensities is a more robust and preferred measure of cleavage than either intensity alone.

[0080] The excitation state lifetime of the donor moiety is, likewise, independent of the absolute amount of substrate, excitation intensity, or turbidity or other background absorbances. Its measurement requires equipment with nanosecond time resolution.

[0081] Fluorescence in a sample is measured using a fluorimeter. In general, excitation radiation, from an excitation source having a first wavelength, passes through excitation optics. The excitation optics cause the excitation radiation to excite the sample. In response, fluorescent proteins in the sample emit radiation which has a wavelength that is different from the excitation wavelength. Collection optics then collect the emission from the sample. The device can include a temperature controller to maintain the sample at a specific temperature while it is being scanned. According to one embodiment, a multi-axis translation stage moves a microtiter plate holding a plurality of samples in order to position different wells to be exposed. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection can be managed by an appropriately programmed digital computer. The computer also can transform the data collected during the assay into another format for presentation.

[0082] Methods of performing assays on fluorescent materials are well known in the art and are described in, e.g.,

Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, New York:Plenum Press (1983); Herman, B., Resonance energy transfer microscopy, in: *Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology*, vol. 30, ed. Taylor, D.L. & Wang, Y.-L., San Diego: Academic Press (1989), pp. 219-243; Turro, N.J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Col, Inc. (1978), pp. 296-361.

[0083] Enzymatic assays also can be performed on living cells *in vivo*, or from samples derived from organisms transfected to express the tandem construct. Because tandem construct fusion proteins can be expressed recombinantly inside a cell, the amount of enzyme activity in the cell or organism of which it is a part can be determined by determining changes in fluorescence of cells or samples from the organism.

[0084] In one embodiment, a cell is transiently or stably transfected with an expression vector encoding a tandem fluorescent protein construct containing a linker moiety that is specifically cleaved by the enzyme to be assayed. This expression vector optionally includes controlling nucleotide sequences such as promotor or enhancing elements. The enzyme to be assayed may either be intrinsic to the cell or may be introduced by stable transfection or transient co-transfection with another expression vector encoding the enzyme and optionally including controlling nucleotide sequences such as promoter or enhancer elements. The fluorescent protein construct and the enzyme preferably are expressed in the same cellular compartment so that they have more opportunity to come into contact.

[0085] If the cell does not possess enzyme activity, the efficiency of FRET in the cell is high, and the fluorescence characteristics of the cell reflect this efficiency. If the cell possesses a high degree of enzyme activity, most of the tandem construct expressed by the cell will be cleaved. In this case, the efficiency of FRET is low, reflecting a large amount or high efficiency of the cleavage enzyme relative to the rate of synthesis of the tandem fluorescent protein construct. If the level of enzyme activity in the cell is such that an equilibrium is reached between expression and cleavage of the tandem construct, the fluorescence characteristics will reflect this equilibrium level. In one aspect, this method can be used to compare mutant cells to identify which ones possess greater or less enzymatic activity. Such cells can be sorted by a fluorescent cell sorter based on fluorescence.

[0086] A contemplated variation of the above assay is to use the controlling nucleotide sequences to produce a sudden increase in the expression of either the tandem fluorescent protein construct or the enzyme being assayed, e. g., by inducing expression of the construct. The efficiency of FRET is monitored at one or more time intervals after the onset of increased expression. A low efficiency or rapid decline of FRET reflects a large amount or high efficiency of the cleavage enzyme. This kinetic determination has the advantage of minimizing any dependency of the assay on the rates of degradation or loss of the fluorescent protein moieties.

[0087] Libraries of host cells expressing tandem fluorescent protein candidate substrates are useful in identifying linker sequences that can be cleaved by a target protease. In general, one begins with a library of recombinant host cells, each of which expresses a different fluorescent protein candidate substrate. Each cell is expanded into a clonal population that is genetically homogeneous. The method consists of measuring FRET from each clonal population before and at least one specified time after a known change in intracellular protease activity. This could be achieved using a fluorimeter, a 96 well plate reader, or by FACS (fluorescence Activated Cell Sorting) anlysis and sorting. This change in protease activity could be produced by transfection with a gene encoding the protease, or infection of a cell by a virus, or induction of protease gene expression using expression control elements, or by any condition that post-translationally modulates the activity of a protease that has already been expressed. An example of the latter is the activation of Calpain 1 by increases in intracellular calcium. The nucleic acids from cells exhibiting a change in FRET can be isolated for example by PCR amplification, and the linker sequences that could be cleaved by the protease identified by sequencing. The results from these studies could used as the basis for the generation of more targeted libraries to identify optimal cleavage motifs through repeated rounds of analysis and selection of clones exhibiting the largest and most rapid changes in FRET in the presence, but not the absence of the protease.

[0088] In another embodiment, the vector may be incorporated into an entire organism by standard transgenic or gene replacement techniques. An expression vector capable of expressing the enzyme optionally may be incorporated into the entire organism by standard transgenic or gene replacement techniques. Then, a sample from the organism containing the tandem construct or the cleaved moieties is tested. For example, cell or tissue homogenates, individual cells, or samples of body fluids, such as blood, can be tested.

[0089] The enzymatic assays of the invention can be used in drug screening assays to identify compounds that alter the activity of an enzyme. In one embodiment, the assay is performed on a sample *in vitro* containing the enzyme. A sample containing a known amount of enzyme is mixed with a tandem construct of the invention and with a test compound. The amount of the enzyme activity in the sample is then determined as above, e.g., by determining the degree of fluorescence at a first and second time after contact between the sample, the tandem construct and the compound. Then the amount of activity per mole of enzyme in the presence of the test compound is compared with the activity per mole of enzyme in the absence of the test compound. A difference indicates that the test compound alters the activity of the enzyme.

[0090] In another embodiment, the ability of a compound to alter enzyme activity *in vivo* is determined. In an *in vivo* assay, cells transfected with a expression vector encoding a tandem construct of the invention are exposed to different

amounts of the test compound, and the effect on fluorescence in each cell can be determined. Typically, the difference is calibrated against standard measurements to yield an absolute amount of enzyme activity. A test compound that inhibits or blocks the expression of the enzyme can be detected by increased FRET in treated cells compared to untreated controls.

[0091] The following examples are offered by way of illustration, not by way of limitation.

EXAMPLES

EXAMPLE 1: Construction of Tandem Fluorescent Protein Constructs

[0092] Mutant Green Fluorescent Proteins were created as follows. Random mutagenesis of the *Aequorea* green fluorescent protein (**FIG. 1**) was performed by increasing the error rate of the PCR with 0.1 mM $MnCl_2$ and unbalanced nucleotide concentrations. The templates used for PCR encoded the GFP mutants S65T, Y66H and Y66W. They had been cloned into the BamHI site of the expression vector pRSETB (Invitrogen), which includes a T7 promoter and a polyhistidine tag. The GFP coding region (shown in bold) was flanked by the following 5' and 3' sequences: 5'-G GAT CCC CCC GCT GAA TTC **ATG** (SEQ ID NO:19) ... **AAA TAA** TAA GGA TCC (SEQ ID NO:20) -3'. The 5' primer for the mutagenic PCR was the T7 primer matching the vector sequence; the 3' primer was 5'-GGT AAG CTT TTA TTT GTA TAG TTC ATC CAT GCC-3' (SEQ ID NO:21), specific for the 3' end of GFP, creating a HindIII restriction site next to the stop codon.

[0093] Amplification was over 25 cycles (1 min at 94°C, 1 min 52°C, 1 min 72°C) using the AmpliTaq polymerase from Perkin Elmer). Four separate reactions were run in which the concentration of a different nucleotide was lowered from 200 µM to 50 µM. The PCR products were combined, digested with BamHI and HindIII and ligated to the pRSETB cut with BamHI and HindIII. The ligation mixture was dialyzed against water, dried and subsequently transformed into the bacterial strain BL21(DE3) by electroporation (50 µl electrocompetent cells in 0.1 cm cuvettes, 1900 V, 200 ohm, 25 µF). Colonies on agar were visually screened for brightness as previously described. R. Heim et al., "Wavelength mutations and post-translational autooxidation of green fluorescent protein," *Proc Natl Acad Sci USA* 1994, 91: 12501-12504. On the order of 7000 colonies were examined in each successful round of mutagenesis, which is not claimed to be exhaustive. The selected clones were sequenced with the Sequenase version 2.0 kit from United States Biochemical.

[0094] A nucleic acid sequence encoding a tandem GFP-BFP construct fusion protein was produced as follows. The DNA of the GFP mutant S65C (Heim R, Cubitt AB, Tsien RY, "Improved green fluorescence," *Nature* 1995, 373:663-664) was amplified by PCR (1 cycle 3 min 94°C, 2 min 33°C, 2 min 72°C; 20 cycles 1 min 94°C, 1 min 44°C, 1 min 72°C) with Pfu polymerase (Stratagene) using the primers 5'-AGA AAG GCT AGC AAA GGA GAA GAA C-3' (SEQ ID NO: 22) and 5'-T CAG TCT AGA TTT GTA TAG TTC ATC-3' (SEQ ID NO:23) to create a NheI site and a (NheI compatible) XbaI site and to eliminate the GFP stop codon. The restricted product was cloned in-frame into the NheI site of the construct pRSETB-Y66H/Y145F, between a polyhistidine tag and an enterokinase cleavage site. When translated this fusion gives the following sequence: MRGSHHHHHH GMA (SEQ ID NO:24) -- (S2...GFP:S65C...K238 "S65C") -- SSMTGGQQMG RDLYDDDDKD PPAEF (SEQ ID NO:25) -- (GFP:Y66H/Y145F "P4-3"). The linker moiety includes cleavage recognition sites for many proteases, including trypsin, enterokinase and calpain:

```
                calpain
                   /
SSMTGGQQMG RDLYDDDDKD PPAEF (SEQ ID NO:25)
           /       /
      trypsin   trypsin, enterokinase
```

Several other constructs were constructed and tested using the same linker moiety. One of these has the structure S65C-- linker -- P4. Another had the structure S65C -- linker -- W7. A third construct had the structure S65T -- linker -- W7. A fourth construct had the structure P4-3 -- linker -- W7.

[0095] Cultures with freshly transformed *E. coli* cells were grown at 37°C to an optical density of 0.8 at 600 nm, then induced with 0.4 mM isopropylthiogalactoside overnight at room temperature. Expression levels were roughly equivalent between mutants and are typical for the T7 expression system used. Cells were washed in PBS pH 7.4, resuspended in 50 mM Tris pH 8.0, 300 mM NaCl and lysed in a French press. The polyhistidine-tagged GFP proteins were purified from cleared lysates on nickel-chelate columns (Qiagen) using 100 mM imidazole in the above buffer to elute the protein. Samples used for proteolytic experiments were further purified by MonoQ FPLC to remove monomeric GFP. Protein concentrations were estimated with bicinchoninic acid (BCA kit from Pierce) using bovine serum albumin

as a standard.

EXAMPLE 2: Cleavage Measurements

[0096] Proteolytic cleavage of 10 µg of the various GFP-BFP fusion proteins were performed in 500 µl PBS pH 7.4 with 0.1 µg trypsin (Sigma, grade III) and emission spectra were recorded at different time intervals. Analogous cleavage experiments were done also with enterokinase (Sigma) and calpain.

[0097] Excitation spectra were obtained by collecting emission at the respective peak wavelengths and were corrected by a Rhodamine B quantum counter. Emission spectra were likewise measured at the respective excitation peaks and were corrected using factors from the fluorometer manufacturer (Spex Industries, Edison, NJ). In cleavage experiments emission spectra were recorded at excitation 368 nm or at 432 nm. For measuring molar extinction coefficients, 20 to 30 µg of protein were used in 1 ml of PBS pH 7.4. The extinction coefficients in **TABLE I** necessarily assume that the protein is homogeneous and properly folded; if this assumption is incorrect, the real extinction coefficients could be yet higher. Quantum yields of wild-type GFP, S65T, and P4-1 mutants were estimated by comparison with fluorescein in 0.1 N NaOH as a standard of quantum yield 0.91. J.N. Miller, ed., *Standards in Fluorescence Spectrometry,* New York: Chapman and Hall (1981). Mutants P4 and P4-3 were likewise compared to 9-aminoacridine in water (quantum yield 0.98). W2 and W7 were compared to both standards, which gave concordant results.

[0098] Excited at 368 nm, the uncleaved S65C -- linker -- P4-3 construct emitted bright green light that gradually dimmed upon cleavage of the linker to separate the protein domains. As the cleavage by trypsin progressed (0, 2, 5, 10, and 47 min), more blue light was emitted. There was no further change after 47 minutes.

[0099] The emission spectrum of the intact fusion protein (**FIG. 3**) shows that FRET is fairly efficient, because UV excitation causes substantial green emission from the acceptor **S65C**. After proteolytic cleavage of the spacer, which permits the two domains to diffuse apart, the green emission almost completely disappears, whereas the blue emission from the Y66H/Y145F is enhanced because its excited state is no longer being quenched by the acceptor. Control experiments with the same proteolytic conditions applied to either GFP mutant alone showed no effect, arguing that the GFP domains *per se* are resistant to proteolysis, as is known to be the case for the native protein. W.W. Ward et al., "Spectral perturbations of the *Aequorea* green-fluorescent protein," *Photochem. Photobiol.* (1982) 35:803-808.

[0100] Similar result were obtained when the S65C -- linker -- P4-3 fusion construct was cleaved with calpain and excited at 368 nm. (See **FIG.** 4.)

[0101] The tandem construct S65C -- linker -- P4 was exposed to enterokinase and excited at 368 nm. FRET diminished over time, demonstrating that one could detect cleavage of the linker by enterokinase. (See **FIG.** 5.)

[0102] The tandem construct S65T -- linker -- W7 was exposed to trypsin and excited at 432 nm. Cleavage of the linker and separation of the moieties was detectable as a decrease in FRET over time. (See **FIG.** 6.)

[0103] The tandem construct P4-3 -- linker -- W7 was exposed to trypsin and excited at 368 nm. **FIG.** 7. demonstrates the change in FRET resulting from cleavage.

[0104] The tandem construct W1B -- linker -- 10c was exposed to trypsin and excited at 433 nm. **FIG.** 8. demonstrates the change in FRET resulting from cleavage.

[0105] **FIG.** 9 depicts fluorescent ratio changes upon cleavage of a composition containing the tandem construct W1B -- linker -- 10c fluorescent construct at different protein concentrations after exposure to trypsin measured in a fluorescent 96 well microtitre plate reader (a CytoFluor II Series 4000 Perseptive Biosystems. Microtitre wells were excited with light at 395+/-25 nm, and the emitted light measured at 460+/-20 nm and 530+/-15 nm using appriopriate excitation and emission filter sets.

[0106] These different tandem fluorescent protein constructs demonstrate that fluorescence resonance energy transfer can monitor the distance between fluorescent protein domains. Disruption of FRET between man-made chromophores in a short synthetic peptide has been used before to assay proteases (G.A. Krafft et al., "Synthetic approaches to continuous assays of retroviral proteases," *Methods Enzymol.* (1994) 241:70-86; C.G. Knight, "Fluorimetric assays of proteolytic enzymes," *Methods Enzymol.* (1995) 248:18-34), but use of fluorescent proteins as the fluorophores gives the unique possibility of replacing organic synthesis by molecular biology and monitoring proteases *in situ* in living cells and organisms. FRET is also one of the few methods for imaging dynamic non-covalent protein-protein associations *in situ.*

SEQUENCE LISTING

[0107]

(1) GENERAL INFORMATION:

(i) APPLICANT: Tsien, Roger Y. Heim, Roger

(ii) TITLE OF INVENTION: Tandem Fluorescent Protein Constructs

(iii) NUMBER OF SEQUENCES: 25

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
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(C) CLASSIFICATION:

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(ix) TELECOMMUNICATION INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 717 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..717

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT
   48
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
 1               5                  10                  15

GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG
   96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30

GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC
  144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45

ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT TTC
  192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60
```

```
TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG
  240
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
 65                  70                  75                  80

CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA
  288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                 85                  90                  95

ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC
  336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110

AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT
  384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125

GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC
  432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
    130                 135                 140
```

```
TAT AAC TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA
    480
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160

ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA AGC GTT
   528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175

CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT
   576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190

GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG
  624
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
        195                 200                 205

AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA
   672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
    210                 215                 220

ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA TA
717
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 238 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
 1               5                  10                  15

Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30

Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45

Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60

Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
65                  70                  75                  80

His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95

Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110

Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125

Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
    130                 135                 140

Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160

Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175

Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190

Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
        195                 200                 205

Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
    210                 215                 220

Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Ser Gln Asn Tyr Pro Ile Val Gly
1               5
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Lys Ala Arg Val Leu Ala Glu Ala Met Ser
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Pro Ser Pro Arg Glu Gly Lys Arg Ser Tyr
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Val Ala Asp Gly
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Phe Gly Gly Ala Lys Lys Arg
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Val Val Asn Ala Ser Ser Arg Leu Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Ile Ala Tyr Leu Lys Lys Ala Thr
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Val Lys Met Asp Ala Glu Phe
1               5
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Phe Leu Ala Glu Gly Gly Gly Val Arg Gly Pro Arg Val Val Glu
Arg
1               5                   10                  15

His
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Asp Arg Val Tyr Ile His Pro Phe His Leu Val Ile His
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Lys Pro Ala Leu Phe Phe Arg Leu
1               5
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Gln Pro Leu Gly Gln Thr Ser Leu Met Lys Arg Pro Pro Gly Phe
Ser
1               5                   10                  15

Pro Phe Arg Ser Val Gln Val Met Lys Thr Gln Glu Gly Ser
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Gly Gly Gly Gly Ser
1               5
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Gly Gly Gly Gly Gly Gly Ser Met Phe Gly Gly Ala Lys Lys Arg
Ser
1               5                   10                  15

Gly Gly Gly Gly Gly Gly
            20
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Ile Gln Arg Met Lys Gln Leu Glu Asp Lys Val Glu Glu Leu Leu
Ser
1               5                   10                  15

Lys Asn Tyr His Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu
Val
            20                  25                  30

Gly Glu Arg
        35
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Ser Lys Val Ile Leu Phe
1               5
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGATCCCCCC GCTGAATTCA TG
22

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAATAATAAG GATCC
15

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTAAGCTTT TATTTGTATA GTTCATCCAT GCC
33

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGAAAGGCTA GCAAAGGAGA AGAA
24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCAGTCTAGA TTTGTATAGT TCATC
25

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Arg Gly Ser His His His His His His
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Ser Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp
Asp
1               5                   10                  15

Asp Asp Lys Asp Pro Pro Ala Glu Phe
            20              25→
```

SEQ. ID. No.: 26

```
12
Ala Asn Pro Leu Tyr Lys Asp Ala Thr Asp Phe
```

SEQ. ID. No.: 27

```
14
Thr Ala Asn Pro Leu Tyr Lys Asp Ala Thr Ser Asp Phe
```

SEQ. ID. No.: 28

```
16
Gly Thr Ala Asn Pro Leu Tyr Lys Asp Ala Thr Ser Gly Asp Phe
```

SEQ. ID. No.: 29

```
18
Gly Thr Ala Asn Pro Leu Tyr Lys Asp Ala Thr Ser Gly Ser Thr
Asp Phe
```

SEQ. ID. No.: 30

```
20
Gly Thr Ala Asn Pro Leu Tyr Lys Asp Ala Thr Ser Gly Ser Thr
Gly Ser Asp Phe
```

SEQ. ID. No.: 31

22
Gly Thr Ala Asn Pro Leu Tyr Lys Asp Ala Thr Ser Gly Ser Thr
Gly Ser Gly Ser Asp Phe

Claims

1. A tandem fluorescent protein construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties and wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer ("FRET") when the donor moiety is excited by radiation, **characterized in that** the linker moiety comprises a protease cleavage recognition site, and wherein cleavage of the linker by a protease results in a change in FRET between the donor and acceptor moieties,
and wherein the donor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acid substitutions,

   a) Phe64Leu, Ser65Thr, Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala and Asn212Lys, or
   b) Ser65Gly, Val68Leu, Ser72Ala and Thr203Tyr, or
   c) Tyr66His and Tyr145Phe, or
   d) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala and Asn212Lys,

   and
   the acceptor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acids substitutions,

   a) Ser65Gly, Val68Leu, Ser72Ala and Thr203Tyr, or
   b) Ser65Thr, Ser72Ala, Asn149Lys, Met153Thr and Ile167Thr.

2. A tandem fluorescent protein construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties and wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer ("FRET") when the donor moiety is excited by radiation, **characterized in that** the linker moiety comprises a protease cleavage recognition site, and wherein cleavage of the linker by a protease results in a change in FRET between the donor and acceptor moieties,
and wherein the donor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acid substitutions,

   a) Tyr66His and Tyr145Phe, or
   b) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala and Asn212Lys,

   and
   the acceptor moiety comprises an *Aequorea* fluorescent protein (SEQ ID NO:2) comprising the amino acids substitutions,

   a) Ser65Cys, or
   b) Ser65Thr.

3. The construct of claim 1 or 2, wherein the linker moiety comprises between 5 amino acids and 50 amino acids, preferably between 10 amino acids and 30 amino acids.

4. The construct of claim 1 or 2, comprising a fusion protein including the donor moiety, the acceptor moiety and the linker moiety in a single amino acid sequence.

5. The construct of claim 1 to 4, wherein the linker moiety comprises a cleavage recognition site for trypsin, enterokinase, HIV-1 protease, prohormone convertase, interleukin-1b-converting enzyme, adenovirus endopeptidase, cytomegalovirus assemblin, leishmanolysin, b-Secretase for APP, thrombin, renin, angiotensin-converting enzyme,

cathepsin D or a kininogenase.

6. The construct of claim 4, wherein the donor is positioned at the amino terminus of the polypeptide relative to the acceptor moiety.

7. A recombinant nucleic acid coding for expression of a construct of claim 4 to 6 comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety in a single amino acid sequence, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer ("FRET") when the donor moiety is excited by radiation.

8. An expression vector comprising expression control sequences operatively linked to a nucleic acid sequence according to claim 7.

9. An expression vector of claim 8 adapted for function in a prokaryotic or eukaryotic cell.

10. A host cell transfected with an expression vector according to claim 8 to 11.

11. The cell of claim 10 further comprising a protease that is not normally expressed by said cell.

12. The host cell of claim 11 that is a cultured mammalian cell.

13. A method for determining whether a sample contains a protease comprising:

contacting the sample with a construct according to any one of claims 1 to 6, and
exciting the donor moiety by radiation; and
determining the degree of fluorescence resonance energy transfer ("FRET") in the sample, whereby cleavage of the linker moiety through the presence of a protease decreases the degree of fluorescence resonance energy transfer compared to a sample where this activity is not present.

14. The method of claim 13 for determining the amount of a protease in a sample, wherein determining the degree of fluorescence resonance energy transfer ("FRET") in the sample comprises determining the degree at a first and second time after contacting the sample with the construct, and determining the difference in the degree of fluorescence resonance energy transfer, whereby the difference in the degree of fluorescence resonance energy transfer reflects the amount of protease in the sample.

15. The method of claim 13, wherein the step of determining the degree of fluorescence resonance energy transfer ("FRET") in the sample comprises determining the amount of fluorescence from the donor moiety, or determining the amount of fluorescence from the acceptor moiety, or determining the ratio of the amount of fluorescence from the donor moiety and the amount of fluorescence from the acceptor moiety, or determining the excitation state lifetime of the donor moiety.

16. A method of determining the amount of activity of a protease in a cell comprising the steps of:

providing a cell according to any one of claims 10 to 12 that expresses a construct of any one of claims 4 to 6;
exciting the donor moiety by radiation; and
determining the degree of fluorescence resonance energy transfer ("FRET") in the cell, whereby cleavage of the construct by an enzyme results in less fluorescence resonance energy transfer ("FRET") which reflects a protease activity.

17. The method of claim 16, wherein the step of providing a cell comprises inducing expression of the recombinant nucleic acid of claim 7 to produce a sudden increase in expression, and the step of determining the degree of fluorescence resonance energy transfer ("FRET") comprises determining the degree at a first and a second time after expression and determining the difference between the first and second time, whereby less fluorescence resonance energy transfer reflects the presence of a protease.

18. A method of determining the amount of activity of a protease in a sample from an organism having a cell according to any one of claims 10 to 12, comprising the steps of exciting the donor moiety by radiation; and
determining the degree of fluorescence resonance energy transfer ("FRET") in the sample, whereby the higher

the degree of fluorescence resonance energy transfer the lower is the amount of protease activity in the cell.

19. A method for determining whether a compound of interest alters the activity of a protease comprising the steps of:

    contacting a sample containing a known amount of the protease with the compound of interest and with the construct according to any one of claims 1 to 6,
    exciting the donor moiety by radiation; and
    determining the presence of protease activity by a fluorescence property of the sample as a function of the degree of fluorescence resonance energy transfer in the sample.

20. The method of claim 19 wherein the compound of interest is at a predetermined concentration of at least 1 μM.

21. The method of claim 19 further comprising the step of comparing the amount of activity in the sample with a standard activity for the same amount of the protease, whereby a difference between the amount or protease activity in the sample and the standard activity indicates that the compound of interest alters the activity of the protease.

22. A method for determining whether a compound of interest alters the activity of a protease in a cell comprising the steps of:

    providing first and second cells according to claim 10 that express the construct;
    contacting the first cell with an amount of the compound of interest;
    contacting the second cell with a different amount of the compound of interest;
    exciting the donor moiety in the first and second cell by radiation;
    determining the degree of fluorescence resonance energy transfer in the first and second cells; and comparing the degree of fluorescence resonance energy transfer in the first and second cells, whereby a difference in the degree of fluorescence resonance energy transfer indicates that the compound of interest alters the activity of the protease.

23. The method of claim 16, wherein the cleavage enzyme is contacted with the construct by expressing the cleavage enzyme and the construct in a cell.

24. The method of claim 16, wherein the cleavage enzyme is expressed using an inducible promotor and optionally exposing the cell to an inducer of the inducible promotor for less than two hours, wherein the cleavage enzyme is transiently expressed.

25. The method of claim 16, wherein the cleavage enzyme and the construct have a signal sequence directing expression of protein into a vesicle.

26. The method of claim 16, wherein the cell is part of a library of individual clones, wherein said clones have been transfected with nucleic acid sequences coding for constructs having different protease cleavage recognition sites.

27. The use of the constructs according to claim 1 to 6 in protease assays based on the determination of the degree of "FRET" in a sample.

Patentansprüche

1. Ein fluoreszierendes Tandem-Proteinkonstrukt, umfassend einen fluoreszierenden Donorprotein-Teil, einen fluoreszierenden Akzeptorprotein-Teil und einen Linker-Teil, der die Donor- und Akzeptor-Teile aneinander koppelt und wobei die Donor- und Akzeptor-Teile Fluoreszenz-Resonanzenergietransfer ("FRET") aufweisen, wenn der Donor-Teil durch Bestrahlung angeregt wird, **dadurch gekennzeichnet, dass** der Linker-Teil eine Erkennungsstelle für Proteasespaltung umfasst, und wobei Spaltung des Linkers durch eine Protease zu einer Veränderung von FRET zwischen den Donor- und Akzeptor-Teilen führt,
   und wobei der Donor-Teil ein fluoreszierendes Protein (SEQ ID NO:2) aus *Aequorea* umfasst, umfassend die Aminosäuresubstitutionen,

   a) Phe64Leu, Ser65Thr, Tyr66Trp, Asn146Ile, Met153Thr, Val163A und Asn212Lys, oder

b) Ser65Gly, Val68Leu, Ser72Ala und Thr203Tyr, oder
c) Tyr66His and Tyr145Phe, oder
d) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala und Asn12Lys,

und
wobei der Akzeptor-Teil ein fluoreszierendes Protein (SEQ ID NO:2) aus *Aequorea* umfasst, umfassend die Aminosäuresubstitutionen

a) Ser65Gly, Val68Leu, Ser72Ala und Thr203Tyr, oder
b) Ser65Thr, Ser72Ala, Asn149Lys, Met153Thr und Ile167Thr

2. Ein fluoreszierendes Tandem-Proteinkonstrukt, umfassend einen fluoreszierenden Donorprotein-Teil, einen fluoreszierenden Akzeptorprotein-Teil und einen Linker-Teil, der die Donor- und Akzeptor-Teile aneinander koppelt und wobei die Donor- und Akzeptor-Teile Fluoreszenz-Resonanzenergietransfer ("FRET") aufweisen, wenn der Donor-Teil durch Bestrahlung angeregt wird, **dadurch gekennzeichnet, dass** der Linker-Teil eine Erkennungsstelle für Proteasespaltung umfasst, und wobei Spaltung des Linkers durch eine Protease zu einer Veränderung von FRET zwischen den Donor- und Akzeptor-Teilen führt, und wobei der Donor-Teil ein fluoreszierendes Protein (SEQ ID NO:2) aus *Aequorea* umfasst, umfassend die Aminosäuresubstitutionen,

a) Tyr66His und Tyr145Phe, oder
b) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala und Asn12Lys,

und
wobei der Akzeptor-Teil ein fluoreszierendes Protein (SEQ ID NO:2) aus *Aequorea* umfasst, umfassend die Aminosäuresubstitutionen

a) Ser65Cys, oder
b) Ser65Thr.

3. Konstrukt gemäß Anspruch 1 oder 2, wobei der Linker-Teil zwischen 5 Aminosäuren und 50 Aminosäuren umfasst, bevorzugt zwischen 10 Aminosäuren und 30 Aminosäuren.

4. Konstrukt gemäß Anspruch 1 oder 2, umfassend ein Fusionsprotein, das den Donor-Teil, den Akzeptor-Teil and den Linker-Teil in einer einzigen Aminosäuresequenz einschließt.

5. Konstrukt gemäß Anspruch 1 bis 4, wobei der Linker-Teil eine Spaltungserkennungsstelle für Trypsin, Enterokinase, HIV-1 Protease, Prohormon Konvertase, Interleukin-1b-konvertierendes Enzym, Adenovirus Endopeptidase, Cytomegalovirus Assemblin, Leishmanolysin, b-Secretase für APP, Thrombin, Renin, Angiotensin-konvertierendes Enzym, Cathepsin D oder eine Kininogenase umfasst.

6. Konstrukt gemäß Anspruch 4, wobei der Donor relativ zum Akzeptor-Teil am Aminoterminus des Polypeptids positioniert ist.

7. Eine rekombinante Nukleinsäure, die für die Expression eines Konstrukts gemäß Anspruch 4 bis 6 kodiert, umfassend einen fluoreszierenden Donorprotein-Teil, einen fluoreszierenden Akzeptorprotein-Teil und einen Peptidlinker-Teil in einer einzigen Aminosäuresequenz, wobei die Donor- und Akzeptor-Teile Fluoreszenz-Resonanzenergietransfer ("FRET") aufweisen, wenn der Donor-Teil durch Bestrahlung angeregt wird.

8. Ein Expressionsvektor, umfassend Expressionskontrollsequenzen, die funktionell an eine Nukleinsäuresequenz gemäß Anspruch 7 gekoppelt sind.

9. Ein Expressionsvektor gemäß Anspruch 8, geeignet zur Wirkung in einer prokaryotischen oder eukaryotischen Zelle.

10. Eine Wirtszelle, transfiziert mit einem Expressionsvektor gemäß Anspruch 8 bis 11.

11. Zelle gemäß Anspruch 10, weiter umfassend eine Protease, die normalerweise von dieser Zelle nicht exprimiert wird.

12. Wirtszelle gemäß Anspruch 11, die eine kultivierte Säugerzelle ist.

13. Ein Verfahren zur Bestimmung, ob eine Probe eine Protease enthält, umfassend:

in Kontakt bringen der Probe mit einem Konstrukt gemäß einem der Ansprüche 1 bis 6, und Anregen des Donor-Teils durch Bestrahlung; und
Bestimmen des Grades an Fluoreszenz-Resonanzenergietransfer ("FRET") in der Probe, wobei Spaltung des Linker-Teils durch die Anwesenheit einer Protease den Grad an Fluoreszenz-Resonanzenergietransfer im Vergleich zu einer Probe vermindert, in der diese Aktivität nicht anwesend ist.

14. Verfahren gemäß Anspruch 13 zur Bestimmung der Menge einer Protease in einer Probe, wobei die Bestimmung des Grades an Fluoreszenz-Resonanzenergietransfer ("FRET") in der Probe die Bestimmung des Grades bei einer ersten und zweiten Zeit nach dem in Kontakt bringen der Probe mit dem Konstrukt umfasst, und die Bestimmung des Unterschieds im Grad an Fluoreszenz-Resonanzenergietransfer, wobei der Unterschied des Grades an Fluoreszenz-Resonanzenergietransfer die Menge an Protease in der Probe widerspiegelt.

15. Verfahren gemäß Anspruch 13, wobei der Schritt der Bestimmung des Grades an Fluoreszenz-Resonanzenergietransfer ("FRET") in der Probe die Bestimmung der Menge an Fluoreszenz vom Donor-Teil umfasst, oder die Bestimmung der Menge an Fluoreszenz vom Akzeptor-Teil, oder die Bestimmung des Verhältnisses der Menge an Fluoreszenz vom Donor-Teil and der Menge an Fluoreszenz vom Akzeptor-Teil, oder die Bestimmung der Lebensdauer des Anregungszustandes des Donor-Teils.

16. Ein Verfahren zur Bestimmung der Menge an Aktivität einer Protease in einer Zelle, umfassend die Schritte:

Bereitstellen einer Zelle gemäß einem der Ansprüche 10 bis 12, die ein Konstrukt gemäß einem der Ansprüche 4 bis 6 exprimiert;
Anregen des Donor-Teils durch Bestrahlung; und
Bestimmen des Grades an Fluoreszenz-Resonanzenergietransfer (FRET) in der Zelle, wobei die Spaltung des Konstrukts durch ein Enzym zu weniger Fluoreszenz-Resonanzenergietransfer führt, was eine Protease-Aktivität widerspiegelt.

17. Verfahren gemäß Anspruch 16, wobei der Schritt der Bereitstellung einer Zelle die Induktion der Expression der rekombinanten Nukleinsäure gemäß Anspruch 7 umfasst, um eine plötzliche Steigerung der Expression herzustellen, and der Schritt der Bestimmung des Grades an Fluoreszenz-Resonanzenergietransfer ("FRET") umfasst die Bestimmung des Grades bei einer ersten and zweiten Zeit nach Expression and die Bestimmung des Unterschiedes zwischen der ersten and zweiten Zeit, wobei weniger Fluoreszenz-Resonanzenergietransfer die Anwesenheit einer Protease widerspiegelt.

18. Ein Verfahren zur Bestimmung der Menge an Aktivität einer Protease in einer Probe eines Organismus, der eine Zelle gemäß einem der Ansprüche 10 bis 12 aufweist, umfassend die Schritte:

Anregung des Donor-Teils durch Bestrahlung; und
Bestimmung des Grades an Fluoreszenz-Resonanzenergietransfer ("FRET") in der Probe, wobei je höher der Grad an Fluoreszenz-Resonanzenergietransfer ist, desto niedriger die Menge an Proteaseaktivität in der Zelle.

19. Ein Verfahren zur Bestimmung, ob eine interessierende Verbindung die Aktivität einer Protease verändert, umfassend die Schritte:

in Kontakt bringen einer Probe, die eine bekannte Menge der Protease enthält, mit der interessierenden Verbindung and mit dem Konstrukt gemäß einem der Ansprüche 1 bis 6, Anregen des Donor-Teils durch Bestrahlung; und
Bestimmen der Anwesenheit von Proteaseaktivität durch eine Fluoreszenz-Eigenschaft der Probe als Funktion des Grades an Fluoreszenz-Resonanzenergietransfer in der Probe.

20. Verfahren gemäß Anspruch 19, wobei die interessierende Verbindung in einer vorbestimmten Konzentration von wenigstens 1 µM vorliegt.

21. Verfahren gemäß Anspruch 19, weiter umfassend den Schritt des Vergleichs der Menge an Aktivität in der Probe

mit einer Standardaktivität für die gleiche Menge der Protease, wobei ein Unterschied zwischen der Menge oder Proteaseaktivität in der Probe and der Standardaktivität anzeigt, dass die interessierende Verbindung die Aktivität der Protease verändert.

22. Ein Verfahren zur Bestimmung, ob eine Interessierende Verbindung die Aktivität einer Protease in einer Zelle verändert, umfassend die Schritte:

Bereitstellung erster und zweiter Zellen gemäß Anspruch 10, die das Konstrukt exprimieren; in Kontakt bringen der ersten Zelle mit einer Menge der interessierenden Verbindung;
in Kontakt bringen der zweiten Zelle mit einer unterschiedlichen Menge der interessierenden Verbindung;
Anregen des Donor-Teils in der ersten und zweiten Zelle durch Bestrahlung;
Bestimmen des Grades an Fluoreszenz-Resonanzenergietransfer in den ersten und zweiten Zellen;
und Vergleich des Grades an Fluoreszenz-Resonanzenergietransfer in den ersten und zweiten Zellen, wobei ein Unterschied im Grad des Fluoreszenz-Resonanzenergietransfers anzeigt, dass die interessierende Verbindung die Aktivität der Protease verändert.

23. Verfahren gemäß Anspruch 16, wobei das Spaltungsenzym mit dem Konstrukt durch Expression des Spaltungsenzyms und des Konstrukts in einer Zelle in Kontakt gebracht wird.

24. Verfahren gemäß Anspruch 16, wobei das Spaltungsenzym unter Verwendung eines induzierbaren Promotors und durch optionales Aussetzen der Zelle zu einem Inducer des induzierbaren Promotors für weniger als zwei Stunden exprimiert wird, wobei das Spaltungsenzym transient exprimiert wird.

25. Verfahren gemäß Anspruch 16, wobei das Spaltungsenzym und das Konstrukt eine Signalsequenz aufweisen, welche die Expression des Proteins in ein Vesikel steuert.

26. Verfahren gemäß Anspruch 16, wobei die Zelle Teil einer Bibliothek individueller Klone ist, wobei diese Klone mit Nukleinsäuresequenzen transfiziert wurden, die für Konstrukte kodieren, die verschiedene Erkennungsstellen für Proteasespaltung aufweisen.

27. Verwendung der Konstrukte gemäß Anspruch 1 bis 6 in Protease-Assays, basierend auf der Bestimmung des Grades an "FRET" in einer Probe.

Revendications

1. Construction de protéine fluorescente en tandem, comprenant une fraction protéique fluorescente donneur, une fraction protéique fluorescente accepteur et une fraction lieur qui accouple les fractions donneur et accepteur et dans laquelle les fractions donneur et accepteur présentent un transfert d'énergie de résonance de fluorescence ("FRET") lorsque la fraction donneur est excitée par irradiation, **caractérisée en ce que** la fraction lieur comprend un site de reconnaissance de clivage par protéase, et dans laquelle le clivage du lieur par une protéase a pour résultat une modification de FRET entre les fractions donneur et accepteur,
et dans laquelle la fraction donneur comprend une protéine fluorescente de *Aequorea* (SEQ ID NO:2) comprenant les substitutions d'acides aminés

a) Phe64Leu, Ser65Thr, Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala et Asn212Lys, ou
b) Ser65Gly, Val68Leu, Ser72Ala etThr203Tyr, ou
c) Tyr66His etTyr145Phe, ou
d) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala etAsn212Lys,

et
la fraction accepteur comprend une protéine fluorescente de *Aequorea* (SEQ ID NO:2) comprenant les substitutions d'acides aminés

a) Ser65Gly, Val68Leu, Ser72Ala etThr203Tyr, ou
b) Ser65Thr, Ser72Ala, Asn149Lys, Met153Thr et Ile167Thr.

2. Construction de protéine fluorescente en tandem, comprenant une fraction protéique fluorescente donneur, une

fraction protéique fluorescente accepteur et une fraction lieur qui accouple lés fractions donneur et accepteur et dans laquelle les fractions donneur et accepteur présentent un transfert d'énergie de résonance de fluorescence ("FRET") lorsque la fraction donneur est excitée par irradiation, **caractérisée en ce que** la fraction lieur comprend un site de reconnaissance de clivage par protéase, et dans laquelle le clivage du lieur par une protéase a pour résultat une modification de FRET entre les fractions donneur et accepteur,
et dans laquelle la fraction donneur comprend une protéine fluorescente de *Aequorea* (SEQ ID NO:2) comprenant les substitutions d'acides aminés

a) Tyr66His etTyr145Phe, ou
d) Tyr66Trp, Asn146Ile, Met153Thr, Val163Ala etAsn212Lys,

et
la fraction accepteur comprend une protéine fluorescente de *Aequorea* (SEQ ID NO:2) comprenant les substitutions d'acides aminés

a) Ser65Cys, ou
b) Ser65Thr.

3. Construction selon la revendication 1 ou 2, dans laquelle la fraction lieur comprend entre 5 acides aminés et 50 acides aminés, de préférence entre 10 acides aminés et 30 acides aminés.

4. Construction selon la revendication 1 ou 2, comprenant une protéine de fusion comportant la fraction donneur, la fraction accepteur et la fraction lieur dans une seule séquence d'acides aminés.

5. Construction selon l'une des revendications 1 à 4, dans laquelle la fraction lieur comprend un site de reconnaissance de clivage pour la trypsine, l'entérokinase, la protéase de VIH-1, la prohormone convertase, l'enzyme de conversion de l'interleukine 1b, l'adénovirus endopeptidase, l'assembline de cytomégalovirus, la leishmanolysine, la b-sécrétase pour l'APP, la thrombine, la rénine, l'enzyme de conversion de l'angiotensine, la cathepsine D ou une kininogénase.

6. Construction selon la revendication 4, dans laquelle le donneur est positionné sur l'extrémité amino terminale du polypeptide par rapport à la fraction accepteur.

7. Acide nucléique recombinant codant pour l'expression d'une construction selon l'une des revendications 4 à 6, comprenant une fraction protéique fluorescente donneur, une fraction protéique fluorescente accepteur et une fraction lieur peptidique dans une seule séquence d'acides aminés, tandis que les fractions donneur et accepteur présentent un transfert d'énergie de résonance de fluorescence ("FRET") lorsque la fraction donneur est excitée par irradiation.

8. Vecteur d'expression comprenant des séquences de contrôle d'expression liées de façon opérationnelle à une séquence d'acide nucléique selon la revendication 7.

9. Vecteur d'expression selon la revendication 8, adapté pour fonctionner dans une cellule procaryote ou eucaryote.

10. Cellule hôte transfectée avec un vecteur d'expression selon l'une des revendications 8 à 11.

11. Cellule selon la revendication 10, comprenant en outre une protéase qui n'est pas normalement exprimée par ladite cellule.

12. Cellule hôte selon la revendication 11, qui est une cellule mammalienne cultivée.

13. Procédé pour déterminer si un échantillon contient une protéase, comprenant:

la mise en contact de l'échantillon avec une construction selon l'une quelconque des revendications 1 à 6, et l'excitation de la fraction donneur par irradiation, et
la détermination du degré de transfert d'énergie de résonance de fluorescence ("FRET") dans l'échantillon, le clivage de la fraction lieur du fait de la présence d'une protéase diminuant alors le degré de transfert d'énergie de résonance de fluorescence par comparaison avec un échantillon dans lequel cette activité n'est pas pré-

sente.

14. Procédé selon la revendication 13 pour la détermination de la quantité d'une protéase dans un échantillon, dans lequel la détermination du degré de transfert d'énergie de résonance de fluorescence ("FRET") dans l'échantillon comprend la détermination du degré à un premier et un deuxième instants après la mise en contact de l'échantillon avec la construction, et la détermination de la différence dans le degré de transfert d'énergie de résonance de fluorescence, la différence dans le degré de transfert d'énergie de résonance de fluorescence reflétant alors la quantité de protéase dans l'échantillon.

15. Procédé selon la revendication 13, dans lequel l'étape de détermination du degré de transfert d'énergie de résonance de fluorescence ("FRET") dans l'échantillon comprend la détermination de la quantité de fluorescence provenant de la fraction donneur, ou la détermination de la quantité de fluorescence provenant de la fraction accepteur, ou la détermination du rapport de la quantité de fluorescence provenant de la fraction donneur et de la quantité de fluorescence provenant de la fraction accepteur, ou la détermination de la durée de vie de l'état d'excitation de la fraction donneur.

16. Procédé pour la détermination de la quantité d'activité d'une protéase dans une cellule, comprenant les étapes de:

fourniture d'une cellule selon l'une quelconque des revendications 10 à 12 qui exprime une construction selon l'une quelconque des revendications 4 à 6;
excitation de la fraction donneur par irradiation; et
détermination du degré de transfert d'énergie de résonance de fluorescence ("FRET") dans la cellule, le clivage de la construction par une enzyme ayant alors pour résultat un moindre transfert d'énergie de résonance de fluorescence ("FRET"), qui reflète une activité de protéase.

17. Procédé selon la revendication 16, dans lequel l'étape de fourniture d'une cellule comprend l'induction de l'expression de l'acide nucléique recombinant selon la revendication 7 pour produire une soudaine augmentation de l'expression, et l'étape de détermination du degré de transfert d'énergie de résonance de fluorescence ("FRET") comprend la détermination du degré à un premier et un deuxième instants après l'expression et la détermination de la différence entre le premier et le deuxième instants, une perte de transfert d'énergie de résonance de fluorescence reflétant alors la présence d'une protéase.

18. Procédé pour la détermination de la quantité d'activité d'une protéase dans un échantillon provenant d'un organisme ayant une cellule selon l'une quelconque des revendications 10 à 12, comprenant les étapes de:

excitation de la fraction donneur par irradiation; et
détermination du degré de transfert d'énergie de résonance de fluorescence ("FRET") dans l'échantillon, tandis que plus le degré de transfert d'énergie de résonance de fluorescence est élevé, plus la quantité d'activité de protéase dans la cellule est faible.

19. Procédé pour déterminer si un composé d'intérêt altère l'activité d'une protéase, comprenant les étapes de:

mise en contact d'un échantillon contenant une quantité connue de la protéase avec le composé d'intérêt et avec la construction selon l'une quelconque des revendications 1 à 6,
excitation de la fraction donneur par irradiation; et
détermination de la présence d'activité de protéase par une propriété de fluorescence de l'échantillon en fonction du degré de transfert d'énergie de résonance de fluorescence dans l'échantillon.

20. Procédé selon la revendication 19, dans lequel le composé d'intérêt est à une concentration prédéterminée d'au moins 1 µM.

21. Procédé selon la revendication 19, comprenant en outre l'étape de comparaison de la quantité d'activité dans l'échantillon avec une activité standard pour la même quantité de protéase, une différence entre la quantité d'activité de protéase dans l'échantillon et l'activité standard indiquant alors que le composé d'intérêt altère l'activité de la protéase.

22. Procédé pour déterminer si un composé d'intérêt altère l'activité d'une protéase dans une cellule, comprenant les étapes de:

fourniture de première et deuxième cellules selon la revendication 10 qui expriment la construction;
mise en contact de la première cellule avec une quantité du composé d'intérêt;
mise en contact de la deuxième cellule avec une quantité différente du composé d'intérêt;
excitation de la fraction donneur dans la première et la deuxième cellules par irradiation;
détermination du degré de transfert d'énergie de résonance de fluorescence dans la première et la deuxième cellules;
et comparaison du degré de transfert d'énergie de résonance de fluorescence dans la première et la deuxième cellules, une différence dans le degré de transfert d'énergie de résonance de fluorescence indiquant alors que le composé d'intérêt altère l'activité de la protéase.

23. Procédé selon la revendication 16, dans lequel l'enzyme de clivage est mise en contact avec la construction par expression de l'enzyme de clivage et de la construction dans une cellule.

24. Procédé selon la revendication 16, dans lequel l'enzyme de clivage est exprimée au moyen d'un promoteur inductible et en option exposition de la cellule à un inducteur du promoteur inductible pendant moins de deux heures, tandis que l'enzyme de clivage est exprimée de manière transitoire.

25. Procédé selon la revendication 16, dans lequel l'enzyme de clivage de la construction ont une séquence signal dirigeant l'expression de protéine dans une vésicule.

26. Procédé selon la revendication 16, dans lequel la cellule fait partie d'une bibliothèque de clones individuels, tandis que lesdits clones ont été transfectés avec des séquences d'acide nucléique codant pour des constructions ayant des sites de reconnaissance de clivage par protéase différents.

27. Utilisation des constructions selon l'une des revendications 1 à 6 dans des essais de protéase fondés sur la détermination du degré de "FRET" dans un échantillon.

(xi) SEQUENCE DESCRIPTION:

SEQ ID NO:1:

```
ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT      48
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
 1               5                  10                  15

GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG      96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30

GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC     144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45

ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT TTC     192

Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60

TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG     240
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
65                  70                  75                  80

CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA     288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95

ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC     336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110

AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT     384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125

GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC     432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
    130                 135                 140

TAT AAC TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA     480
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160

ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA AGC GTT     528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175

CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT     576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190

GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG     624
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
        195                 200                 205

AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA     672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
    210                 215                 220

ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA TA          717
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
```

FIGURE 1

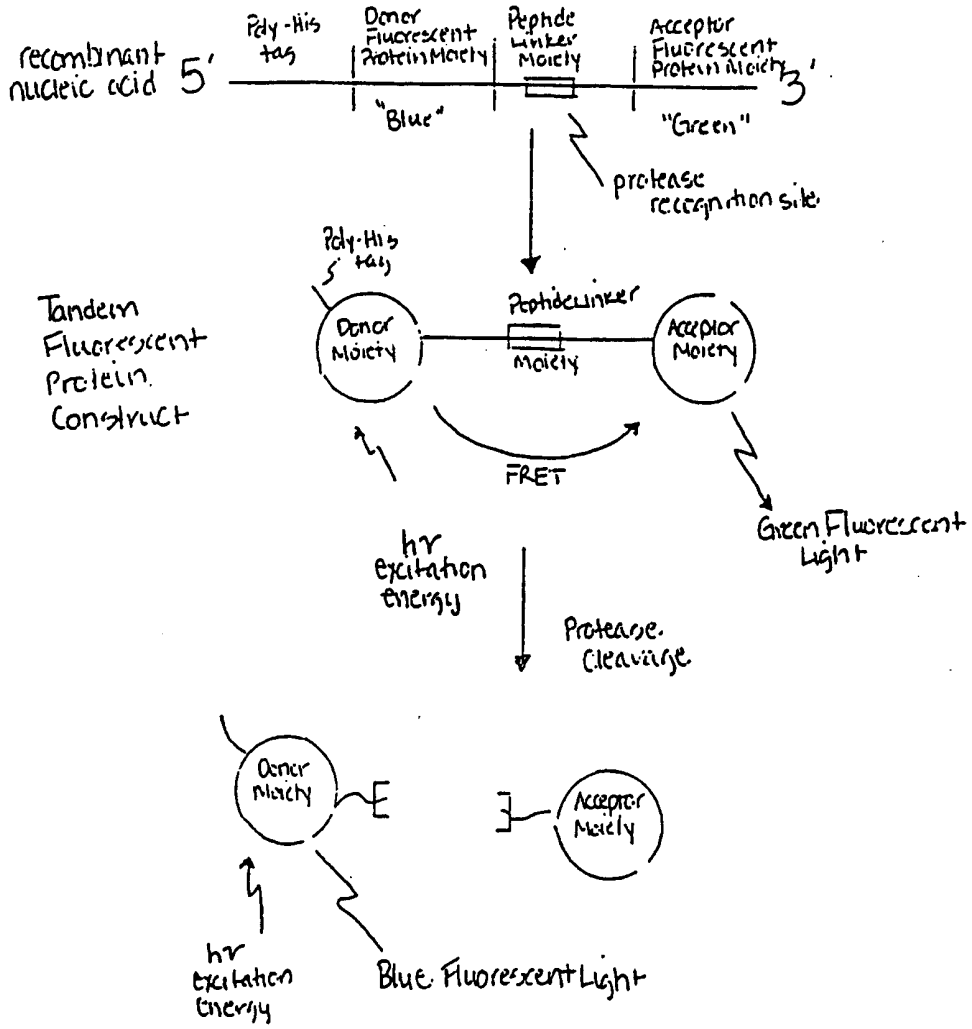
recombinant nucleic acid 5'
Poly-His tag
Donor Fluorescent Protein Moiety
Peptide Linker Moiety
Acceptor Fluorescent Protein Moiety
3'
"Blue"
"Green"
protease recognition site
Tandem Fluorescent Protein Construct
Poly-His 5' tag
Donor Moiety
Peptide Linker Moiety
Acceptor Moiety
FRET
Green Fluorescent Light
hv excitation energy
Protease Cleavage
Donor Moiety
Acceptor Moiety
hv excitation energy
Blue Fluorescent Light

FIG. 2

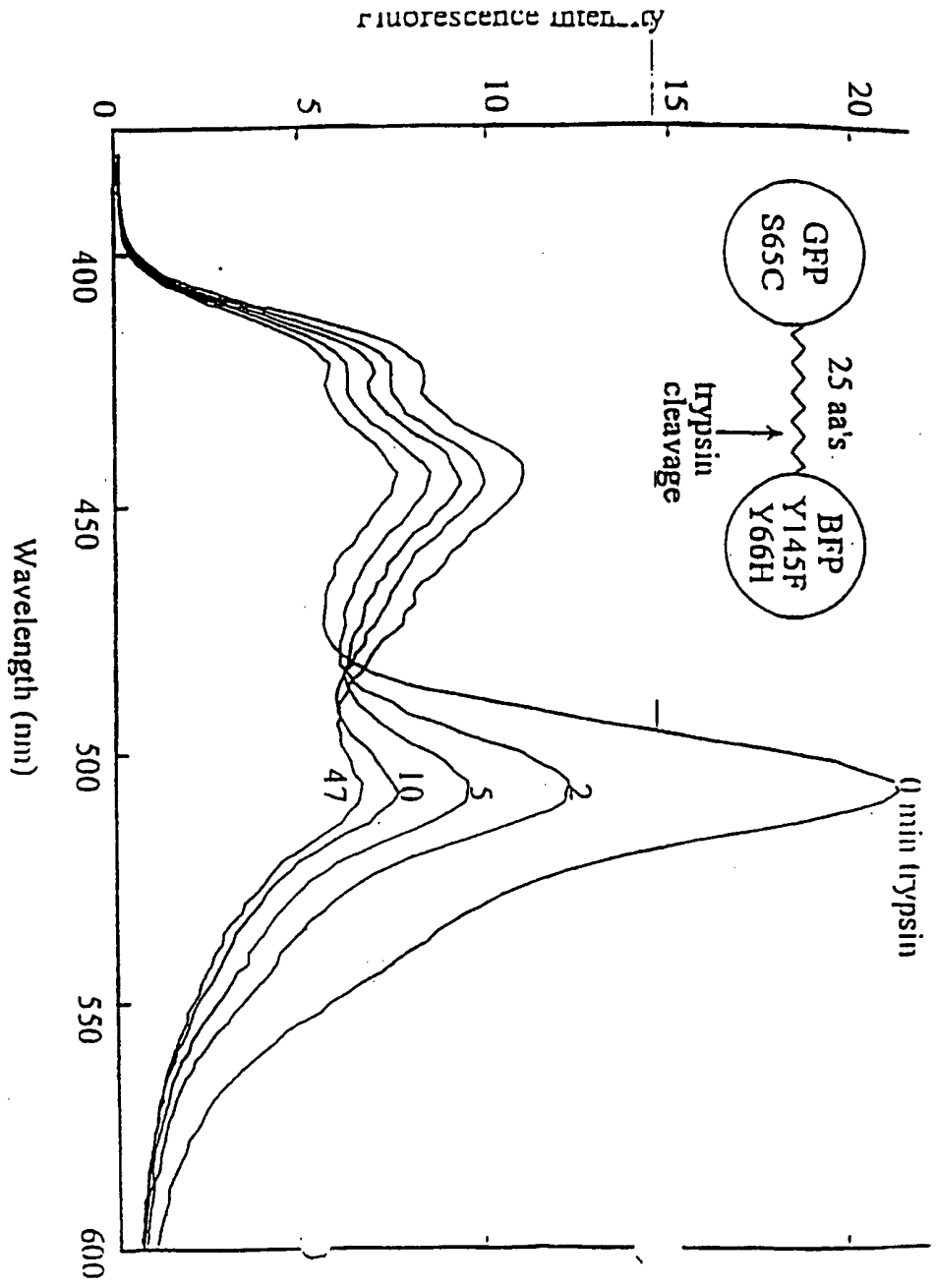
Fluorescence Intensity
0
5
10
15
20
400
450
500
550
600
Wavelength (nm)
GFP
S65C
25 aa's
trypsin
cleavage
BFP
Y145F
Y66H
0 min trypsin
2
5
10
47

FIG. 3

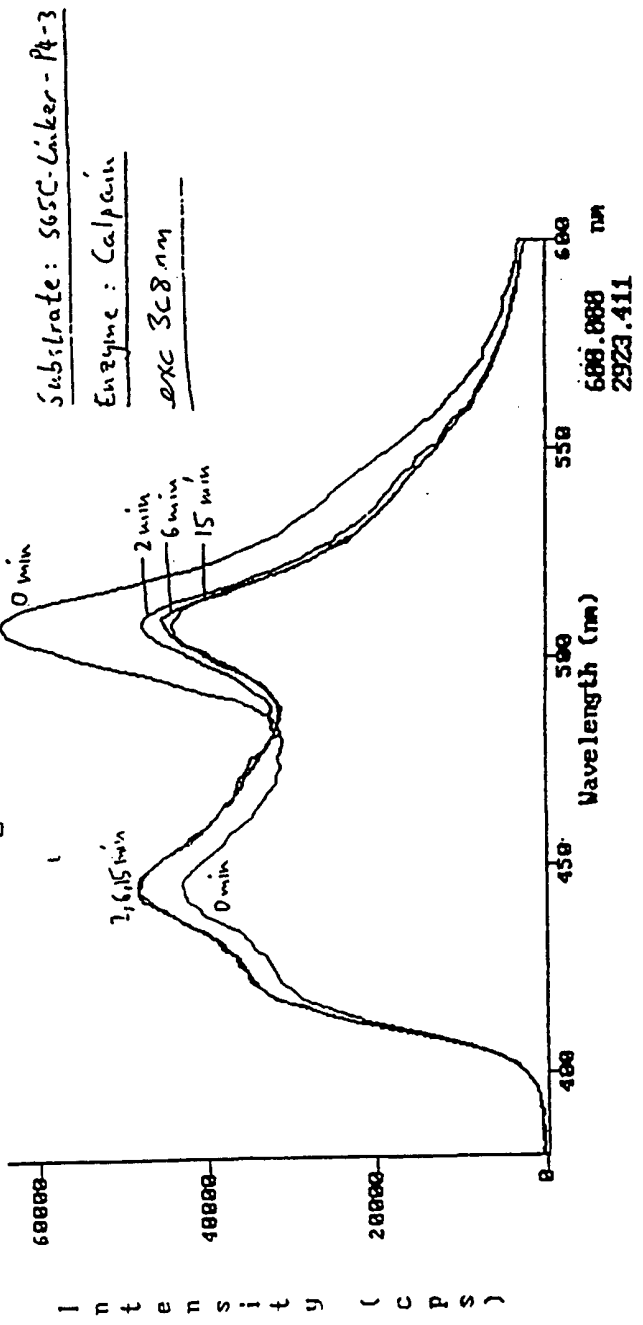
CLC7.SPI
CLC8.SPI
CLC9.SPI
CLC10.SPI
P43/S65T1mMEGTA2ulcalp1B' ex36 11/26/95
P43/S65T1mMCa2ulcalp2' ex36
P43/S65T1mMCa2ulcalp6' ex368
P43/S65T1mMCa2ulcalp15' ex368
Substrate: SGSG-Linker-Pt-3
Enzyme : Calpain
exc 360nm
0 min
2 min
6 min
15 min
1,6,15 min
0 min
Intensity (cps)
Wavelength (nm)
2923.411

FIG. 4

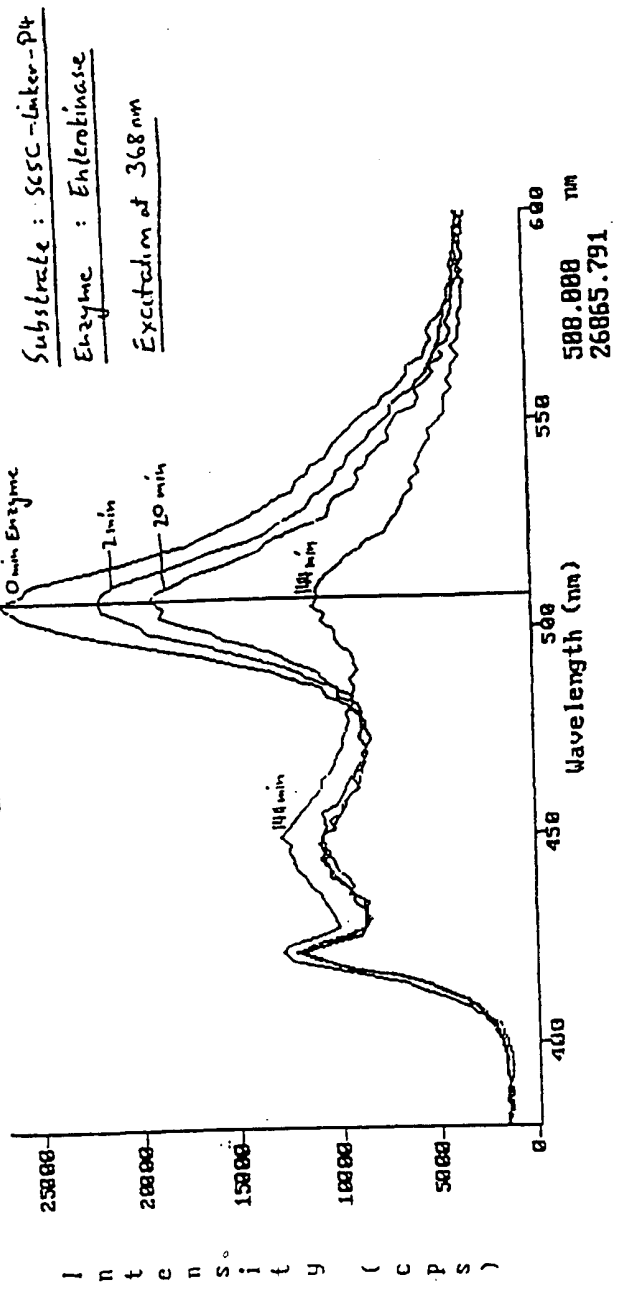
FUSE6.SPI
FUSE5.SPI
FUSE2.SPI
FUSE1.SPI
fusS65C/P4HPLCex368En144'4/12
fusS65C/P4HPLCex368En20'4/12/
fusS65C/P4HPLCex368En2'4/12/9
fusS65C/P4HPLCex368En0'4/12/9
Substrate : SOSC-linker-P4
Enzyme : Enkinase
Excitation at 368 nm
0 min Enzyme
1 min
20 min
144 min
144 min
Intensity (cps)
Wavelength (nm)
500.000
26865.791

FIG.5

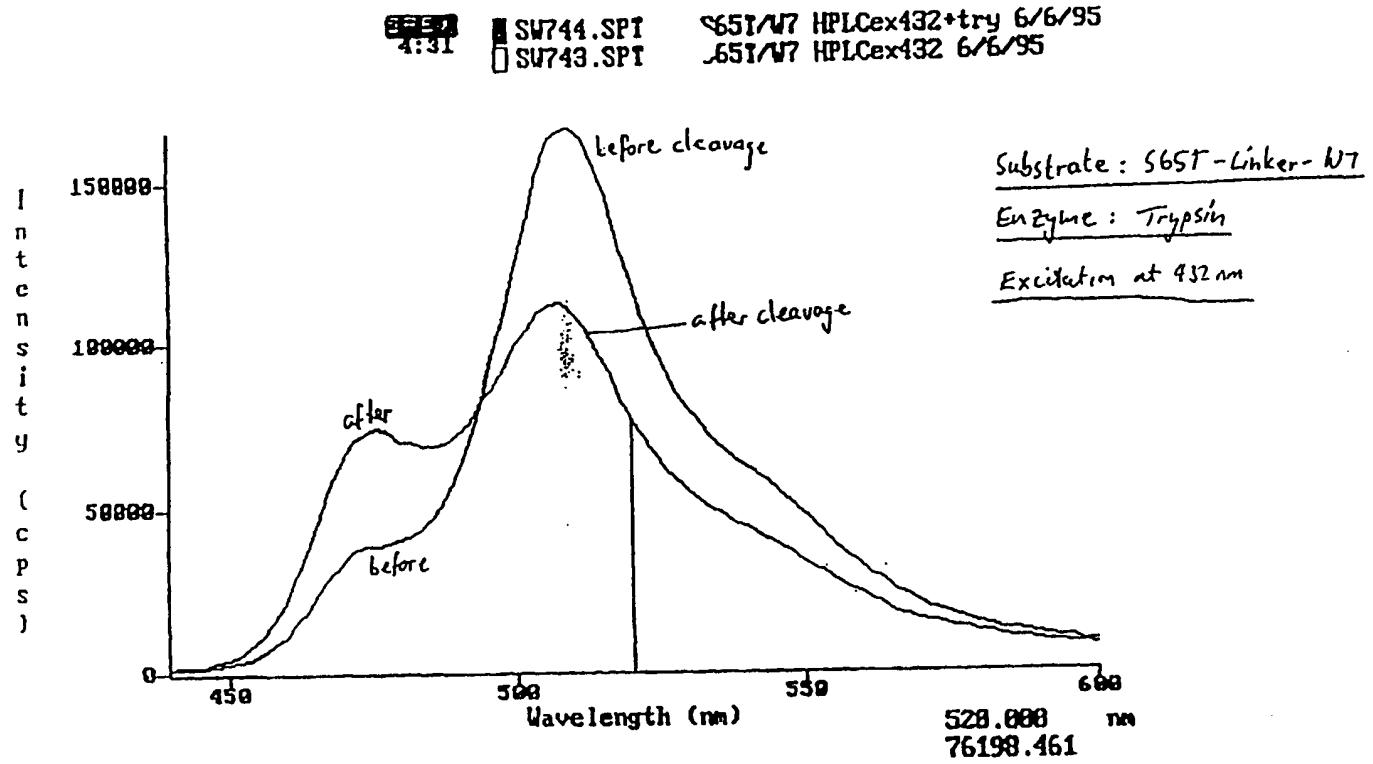

FIG. 6

EP 0 877 805 B1

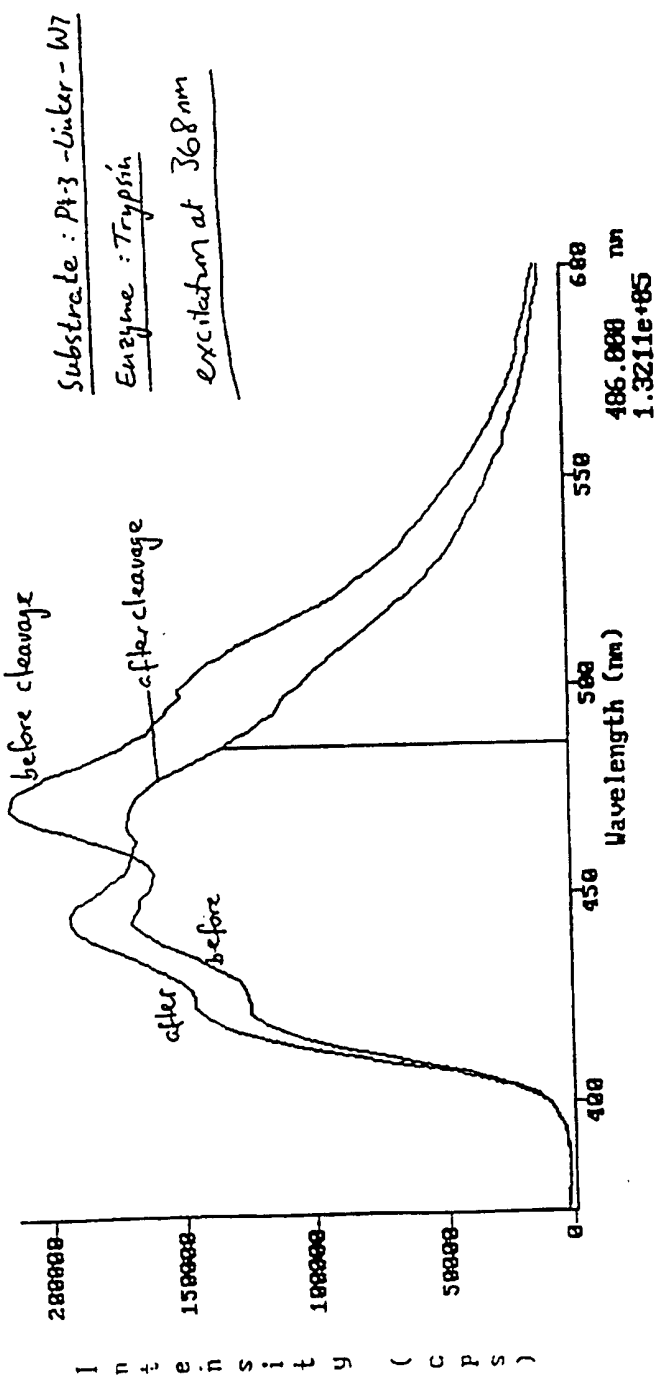
P3W742.SPT
P3W74.SPT
Substrate : P1-3 -Linker - W7
Enzyme : Trypsin
excitation at 368nm
before cleavage
after cleavage
after
before
Intensity (cps)
Wavelength (nm)
400
450
500
550
600
0
100000
150000
200000
486.000 nm
1.3211e+05

FIG. 7

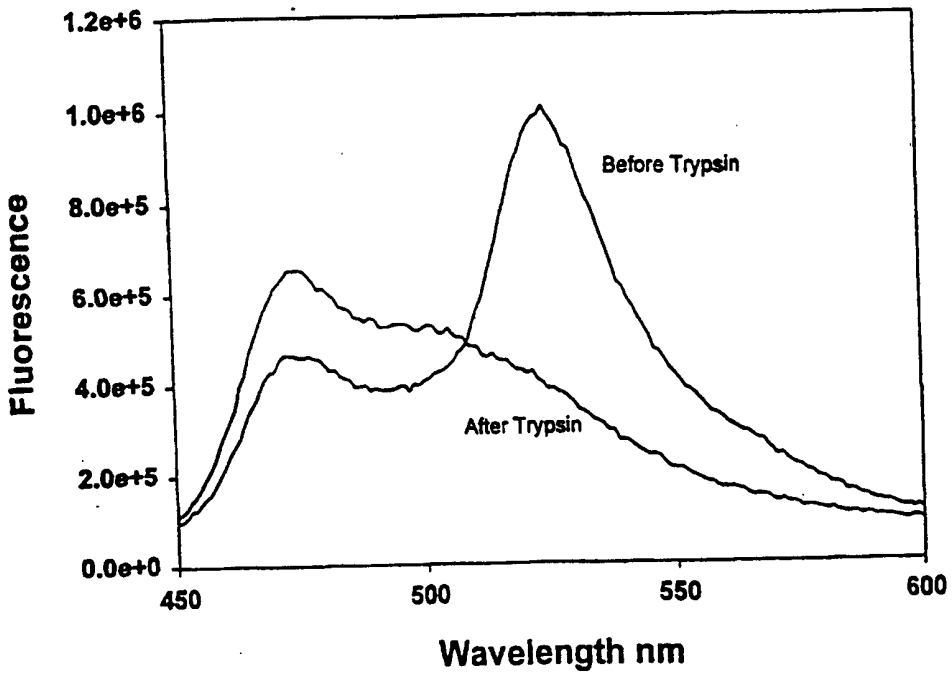
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0e+0
Fluorescence
Before Trypsin
After Trypsin
450
500
550
600
Wavelength nm

FIG. 8

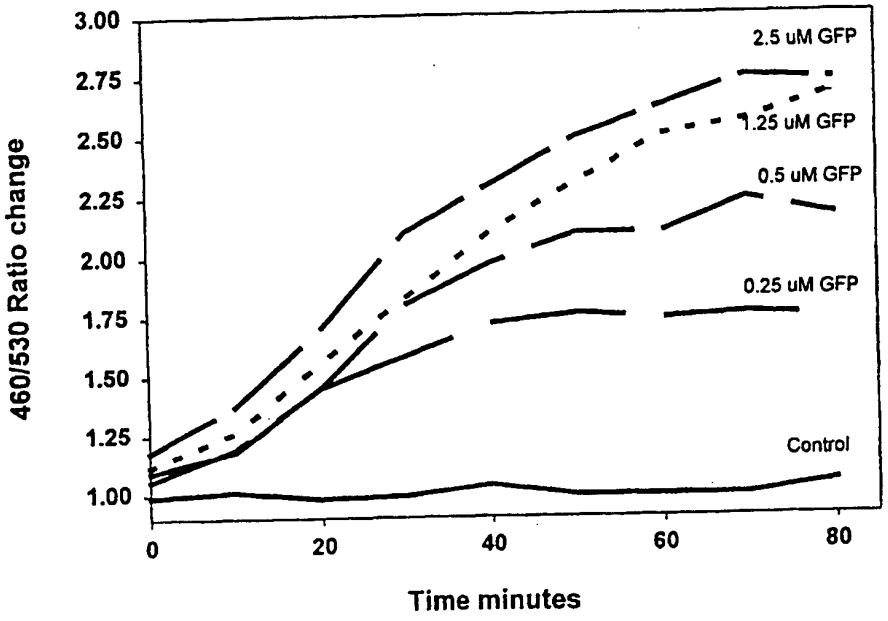
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
460/530 Ratio change
0
20
40
60
80
Time minutes
2.5 uM GFP
1.25 uM GFP
0.5 uM GFP
0.25 uM GFP
Control

FIG. 9